Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 27, SEC. F. DECEMBER, 1949 NUMBER 12

PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

XXXIII. AUTOMATIC pH CONTROL IN THE DISSIMILATION OF SUCROSE BY *BACILLUS POLYMYXA*[1]

BY R. W. WATSON,[2] FLORENCE TAMBOLINE,[3] AND G. W. HARMSEN[3]

Abstract

An electronic control circuit was used to maintain pH within ± 0.02 units between successive additions of soluble alkali. Carbon balances show the effects of a series of pH levels and of a range of sucrose concentrations on the proportional yields of end products. There is a marked change in the chemical balance of this fermentation at about pH 7.0, correlated with a suppression of the acetoin enzyme system. Above pH 6.8 a sharp increase in acid production is correlated with decreased formation of diol and carbon dioxide: below pH 6.8 the yield of organic acids decreases steadily. Most efficient conversion to diol occurs from pH 6.0 to 6.4. Several reasons are advanced for selecting pH 6.2 as the optimum. Under anaerobic conditions the fermentation rate is increased over that under aerobic conditions. Diol yields increase and ethanol yields decrease steadily with increasing sucrose concentrations. The increases in diol are accompanied by decreasing yields of organic acids, and not by changes in carbon dioxide production, which remains relatively uniform. The sucrose concentration most efficient for conversion to diol is about 8%, which is dissimilated anaerobically in 30 hr. at pH 6.2 to yield 65 mM. (millimoles) of diol per 100 mM. of invert sugar fermented. Under aerobic conditions the diol–ethanol ratios show a marked increase, and reach a maximum of about 11 at 10% sucrose. This is due largely to increased acetoin and decreased ethanol formation. The dissimilation of 6% sucrose reaches 98% in 71 hr. under aerobic conditions and yields 82 mM. of diol plus acetoin per 100 mM. of invert sugar fermented. The use of either sodium or potassium hydroxide in place of ammonium hydroxide increases five times the period for complete dissimilation of 5% sucrose. Advantages of controlling the reaction by addition of ammonium hydroxide are reviewed.

Introduction

Continuous precise control of pH at an optimal level by the addition of soluble alkali to fermentation media has been found to increase markedly the rate of carbohydrate dissimilation by bacteria (24, 25, 26, 33). Selection of the optimum pH for the formation of a given product enables a maximum yield to be obtained in a minimum time. It is the purpose of this investigation to establish the optimum pH for the production of D-(*levo*)-2,3-butanediol

[1] *Manuscript received August 31, 1949.*
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as Paper No. 74 on the Industrial Utilization of Wastes and Surpluses and as N.R.C. No. 2032.
[2] *Biochemist, Industrial Utilization Investigations.*
[3] *Bacteriologist, Industrial Utilization Investigations.*

(30) from sucrose and to determine the effects of various sucrose concentrations on fermentation times and product yields under aerobic and anaerobic conditions.

Automatic pH control was first successfully applied to small volumes of fermentation media by Longsworth and MacInnes in their study of the dissimilation of galactose by *Lactobacillus acidophilus* (24). A reliable electronic monitor, differing widely from that used by Longsworth and MacInnes, has recently been produced in these laboratories. The initial impetus in the development of this instrument was provided by Ledingham *et al.* (23). Contributions to its design were made by various members of the Radio and Electrical Engineering Division, and the electronic control circuit has recently been described by Breeze (5, 6). Neish and Ledingham devised accessory apparatus and were the first to use the monitor in studies of the dissimilation of glucose by *Aerobacter aerogenes*, *Bacillus polymyxa*, *Serratia marcescens*, and *Bacillus subtilis* (33). In the belief that the recent three-channel model (6) may be of value in a wide range of chemical and biochemical studies, further information regarding its use is given in this communication.

The production of *levo*-2,3-butanediol by the fermentation of whole wheat mashes, starch, and whole barley has been the subject of numerous investigations. The ability of *B. polymyxa* to ferment starch without preliminary saccharification led to the early utilization of whole wheat (1, 16, 22, 23, 47). Attention was later turned to the use of starch (10, 17, 18) and of ground barley (42). In a further search for more economical source materials, the fermentation of beet molasses has been undertaken in these laboratories on a pilot plant scale. The present study provides information that may serve as a basis for the utilization of molasses.

Experimental

Two locally isolated strains of *B. polymyxa* (C3(2) and C42(3)) and one strain originally obtained from the University of Alberta (UA206) were selected for their high yields of 2,3-butanediol. Studies of the rate of fermentation of 15% whole wheat mashes by C3(2) showed it to be among the four best strains on the basis of diol and ethanol yields (23). Strain C42(3) has given consistently good results with wheat (42) and through the kindness of Mr. F. J. Simpson a promising variant (E13) of C42(3) was obtained from the pilot plant. This variant had been isolated during the course of studies on the adaptation of strains of *B. polymyxa* to the fermentation of molasses, and was selected for its high production of 2,3-butanediol from this substrate.

The complex nitrogen and growth factor requirements of *B. polymyxa* were found by Katznelson and Lochhead (19) to be best supplied by a basic medium containing yeast extract, glucose, and inorganic salts. Reagent sucrose (Merck's and Baker and Adamson's), yeast extract (Difco), and nutrient salt solutions were sterilized in four separate portions to give on mixing 250 ml. of a medium containing varying concentrations of sucrose, 0.5% yeast extract,

0.05% dipotassium hydrogen phosphate, 0.05% potassium dihydrogen phosphate, and 0.02% magnesium sulphate. Addition of 4% inoculum brought the final volume to 260 ml. All fermentations were carried out at 33 ± 1° C.

Inocula prepared in flasks on a shaker started the fermentation more rapidly than those prepared in tubes without agitation (34). Although this effect is helpful in the precision control of pH, in that it allows earlier supervision and necessary adjustment, complete fermentation times and diol yields remain unchanged (4). Aliquots of a sterile stock solution (15 ml. at pH 6.9 to 7.0) containing 1% sucrose and 0.5% yeast extract, inoculated from a 48 hr. agar slant and incubated in 50 ml. Erlenmeyer flasks at 30° C. on a shaker for 16 to 18 hr., gave uniformly good results, and shortened the lag period to two to three hours. The inoculum was examined microscopically for purity and for uniformity before addition to the medium. At the end of each fermentation the maintenance of purity in the culture was established by plating and by the examination of Gram-stained smears.

Procedures in the use of the pH monitor have already been described (5, 6, 33). Supplementary techniques were developed during this investigation to overcome troublesome variations in electrode potential, which sometimes appeared immediately after sterilization of the electrodes by irradiation at λ 2537 in air, and occasionally also during the fermentation. These variations were traced to three main sources: (i) deterioration of the electrode glass, (ii) effects of drying on the bulbs, and (iii) imperfect lead contacts.

Much of the success in the precision control of pH depends on the care given to the glass electrodes. Deterioration begins with use, and the age of a glass electrode may be defined as the length of time it has been in contact with solutions (21). Normal deterioration is probably caused by the escape of sodium or lithium ions from the superficial film. With average care this process limits the useful period for a high-ohmic type (100 to 150 megohms), such as is used with the monitor, to one to two years at 25° C. This period may be lengthened as much as 30% by storage in the proper medium (21). The rate of deterioration approximately doubles for each rise in temperature of 10° C. Sterilization of the glass electrodes by irradiation in air at λ 2537 superimposes on normal aging a more rapid deterioration caused by air drying at an elevated temperature (8). This was eliminated by immersing the bulbs in water contained in dishes of Vycor 791. Electrodes that had become prematurely unusable were readily restored to give precision control by immersing the bulbs for 30 sec. in 10% hydrofluoric acid, followed by several hours in distilled water.

The form and mounting of the electrodes are of critical importance in maintaining cultural purity, and also in the accurate reporting of changes in e.m.f. correlated with pH changes in the flasks. Electrodes with long (7 in.) glass salt-bridge tubes (e.g., Leeds and Northrup 1199–32) may be sealed through reduced 24/40 standard taper joints with high-melting paraffin and polyethylene cement, in a manner that permits sterilization without effecting

any change in the calibration of the bulbs (46). In order to adapt this preferred electrode to a rotating system (6, 33) changes were made in the lead contacts.* These changes not only freed the circuit from fortuitous variations in potential due to faulty contacts, but increased the percentage of successfully controlled fermentations by eliminating losses due to occasional breakages of the leads.

The methods of analysis for 2,3-butanediol, acetoin, ethanol, carbon dioxide, and hydrogen have been previously described (31, 41). Organic acids were determined by partition chromatography (32), and carbon assimilated by wet combustion (33, 45). Residual invert sugar was estimated by the Underkofler semimicro modification of the Shaffer–Somogyi method, standardized with hydrolyzed sucrose (43).

Results

Selection of the most favorable of the common soluble alkalis and of the best strain of *B. polymyxa* preceded establishment of the optimal pH level and of the most suitable sucrose concentration under aerobic and anaerobic conditions. Carbon balances for the anaerobic fermentation of 5% sucrose at pH 7.2 by strain C3(2), in which sodium, potassium, and ammonium hydroxides were used to maintain pH are given in Table I. Use of either sodium or potassium hydroxide in place of ammonium hydroxide retards the fermentation rate so that the period for complete dissimilation is increased more than five times. The yields of ethanol and acetoin are normal for an anaerobic fermentation. The low diol yields are explained by the well known inactivation of the acetoin enzyme system near pH 7.0 (28). Differences between the alkalis become accentuated at a pH as high as 7.2, since much larger amounts must be added for neutralization at this comparatively high pH level. It is difficult to explain the unusually high percentages (169 to 181 mM. per 100 mM. of invert sugar fermented) appearing as carbon assimilated in Table I. These are corrected values and they point to the action of factors other than vigorous growth and the absence of lysis. They may be correlated with polymer formation during carbohydrate degradation.

The response of three different strains to standardized anaerobic conditions in a 4.8% sucrose medium at pH 7.2 is shown in Table II. Strains C3(2) and UA206 display greater sensitivity to continued neutralization of the medium than C42(3)E13. The enzyme system in strain C42(3)E13 responsible for acetoin production apparently becomes inactive only at a higher pH. Compared with normal yields, ethanol production is most markedly increased and diol yield decreased with strain C3(2). The reduced carbon dioxide

*The lead wire from the Standard 1199–32 electrode is removed, the top of the aluminum cap threaded for a ¼ in. stainless steel plug ½ in. long, and bearing upper and lower pins (10 mm. and 5 mm. long respectively) turned from the body of the plug. The aluminum cap is partially filled with mercury, the plug inserted, and a silver-plated beryllium-copper spring clip is attached to the upper pin. A 10 in. double (loop) phosphor-bronze dial cord is soldered to the clip and to the cable leads.

TABLE I

ANAEROBIC DISSIMILATION OF 5% SUCROSE BY STRAIN C3(2) AT pH 7.2 CONTROLLED BY SODIUM, POTASSIUM, AND AMMONIUM HYDROXIDES

Product	Millimoles per 100 mM. invert sugar fermented		
	NaOH	KOH	NH_4OH
2,3-Butanediol	9.0	12.3	11.5
Acetoin	1.2	1.1	3.4
Ethanol	73.5	70.6	73.2
Acetic acid	25.6	22.5	21.1
Formic acid	1.9	1.5	11.7
Succinic acid	0.7	0.5	0.8
Lactic acid	2.3	2.3	1.4
Carbon dioxide	195.3	180.2	150.3
Hydrogen	161.9	144.5	95.8
Carbon assimilated	169.4	169.9	180.7
CO_2 obs./calc.	0.98	0.90	0.75
H_2 obs./calc.	1.62	1.45	0.96
$\frac{\text{Acetoin + diol}}{\text{Ethanol}}$ (by wt.)	0.3	0.4	0.4
Carbon recovery, %	103.0	100.3	100.1
O/R Index	1.15	1.11	1.10
Fermentation time, hr.	119	119	23
% invert sugar used	97.6	92.1	100
pH at beginning	7.20	7.20	7.20
pH at end	7.20	7.24	7.34

TABLE II

ANAEROBIC DISSIMILATION OF 4.8% SUCROSE AT pH 7.2 BY THREE STRAINS OF *Bacillus polymyxa*

Product	Millimoles per 100 mM. invert sugar fermented		
	Strain C3(2)	Strain UA206	Strain C42(3)E13
2,3-Butanediol	18.7	24.1	43.2
Acetoin	6.4	5.4	5.4
Ethanol	82.7	58.6	54.9
Acetic acid	50.4	43.3	23.4
Formic acid	9.1	2.1	14.3
Succinic acid	1.3	0.1	1.9
Lactic acid	2.0	1.6	1.7
Carbon dioxide	162.5	185.8	162.1
Hydrogen	109.3	128.2	75.5
Carbon assimilated	43.4	40.7	44.1
CO_2 obs./calc.	0.81	0.93	0.81
H_2 obs./calc.	1.09	1.28	0.75
$\frac{\text{Acetoin + diol}}{\text{Ethanol}}$ (by wt.)	0.6	1.0	1.7
Carbon recovery	100.0	93.6	98.2
O/R Index	0.96	1.12	1.03
Fermentation time, hr.	22	31	19
% invert sugar used	99.8	99.6	99.2
pH at beginning	7.20	7.20	7.20
Final pH	7.20	7.34	7.43

formation (80 to 90% of theoretical) is correlated with an increase in the production of organic acids.

The apparent sensitivity of strain C3(2) to changing hydrogen ion concentration led to an investigation of its response over a range of pH levels (Table III). To summarize the results, there is a marked change in the chemical balance near pH 7. Diol and ethanol yields become almost normal below

TABLE III

ANAEROBIC DISSIMILATION OF 4.8% SUCROSE AT VARIOUS pH LEVELS BY STRAIN C3(2)

Product	Millimoles per 100 mM. invert sugar fermented					
	pH 5.2	pH 5.6	pH 6.0	pH 6.4	pH 6.8	pH 7.2
2,3-Butanediol	56.0	55.4	50.3	56.3	49.6	18.7
Acetoin	3.0	2.2	2.8	1.2	1.9	6.4
Ethanol	70.3	71.9	57.2	65.4	69.6	82.7
Acetic acid	0.9	1.1	1.3	7.3	19.0	50.4
Formic acid	0.4	0.4	0.5	0.3	0.6	9.1
Succinic acid	0.9	0.6	0.3	0.4	0.3	1.3
Lactic acid	1.0	0.7	1.4	1.5	0.7	2.0
Total organic acids	3.3	2.9	3.6	9.6	20.7	64.0
Carbon dioxide	201.8	181.8	203.8	197.3	193.5	162.5
Hydrogen	102.4	84.7	113.8	102.4	109.0	109.3
Carbon assimilated	27.5	27.1	25.1	29.7	8.8	43.4
CO_2 obs./calc.	1.01	0.91	1.02	0.99	0.97	0.81
H_2 obs./calc.	1.02	0.85	1.14	1.02	1.09	1.09
$\frac{\text{Acetoin + diol}}{\text{Ethanol}}$ (by wt.)	1.6	1.6	1.8	1.7	1.4	0.6
Carbon recovery	102.6	98.5	94.2	101.6	98.4	100.0
O/R Index	0.97	0.91	1.06	0.98	0.98	0.96
Fermentation time, hr.	71	71	29	27	26	22
% invert sugar used	98.4	95.2	91.3	97.2	97.6	99.8
pH at beginning	5.20	5.60	6.00	6.40	6.80	7.20
Final pH	5.27	—	6.19	6.52	7.01	7.20

pH 6.8 while above this level they are suppressed and acids accumulate. This confirms the results obtained by Neish and Ledingham (33) and an observation by Adams and Leslie (2) on pH controlled fermentations of whole wheat mashes by strain C3(2). Production of total and individual acids decreases steadily as the pH falls to 5.6. The marked increase in acid production above pH 6.8 is correlated with a decrease in carbon dioxide formation. Over the entire range from pH 5.2 to 6.8 the production of carbon dioxide remains close to the theoretical. From pH 5.6 to 6.0 there is a conspicuous drop in the time for 95% dissimilation, followed by a slow steady decline as the pH increases to 7.2. Neish and Ledingham (33) using strain C2(1) found the shortest fermentation period to occur at pH values of 6.2 to 6.8. Variations in the percentages of total carbon assimilated are probably associated with the varying degrees of autolysis that occur in an aging culture (23).

For comparison the effects of a range of pH levels on product yields from C42(3)E13 in 4.8% sucrose under similar conditions are shown in Table IV. The general observations apply to the data from both strains. Total yields of diol plus acetoin are somewhat higher at a given pH than with C3(2), although the diol yields themselves are lower. Stahly and Werkman (40) have shown that diol–acetoin ratios are correlated with changes in the oxidation–reduction potential.

TABLE IV

EFFECTS OF pH LEVELS ON PRODUCT YIELDS FROM STRAIN C42(3)E13 IN 4.8% SUCROSE UNDER DEOXYGENATED NITROGEN

Product	Millimoles per 100 mM. invert sugar used			
	pH 6.0	pH 6.4	pH 6.8	pH 7.2
2,3-Butanediol	52.0	53.5	50.4	43.2
Acetoin	4.2	4.9	4.2	5.4
Ethanol	57.6	58.2	56.8	54.9
Acetic acid	4.4	12.2	12.0	23.4
Formic acid	0.7	1.1	0.7	14.3
Succinic acid	0.4	0.4	0.7	1.9
Lactic acid	2.0	2.7	1.6	1.7
Total organic acids	7.8	16.8	15.5	42.2
Carbon dioxide	189.7	176.0	193.5	162.1
Hydrogen	71.8	66.3	88.8	75.5
Carbon assimilated	62.2	44.4	23.8	44.1
CO_2 obs./calc.	0.95	0.88	0.97	0.81
H_2 obs./calc.	0.62	0.66	0.89	0.75
$\frac{\text{Acetoin + diol}}{\text{Ethanol}}$ (by wt.)	1.9	2.0	1.9	1.7
Carbon recovery	101.8	101.4	97.4	98.2
O/R Index	1.08	1.00	1.06	1.03
Fermentation time, hr.	48*	48*	48*	19
% invert sugar used	97.3	97.7	98.3	99.2
pH at beginning	6.00	6.40	6.80	7.20
Final pH	6.12	6.46	6.84	7.43

**Not true fermentation time.*

Complete balances for the anaerobic dissimilation of sucrose in varying concentrations by strain C42(3)E13 at pH 6.2 are given in Table V. Diol yields increase and ethanol yields decrease steadily with increasing sucrose concentration. Up to 8% of sucrose, increases in diol yield are accompanied by decreasing accumulations of acetic acid (28, 35) and slight increases in the production of lactic acid. This is confirmation of the data presented by Mickelson and Werkman (28) and by Reynolds and Werkman (35). The total organic acids produced per 100 mM. of invert sugar fermented decreases at first rapidly and then more slowly with increasing sucrose concentrations (to 8%). Moreover there is a general but irregular increase in fermentation time as the percentage of sucrose in the medium increases (*vide infra*). The most efficient conversion to diol occurs in media containing about 8% sucrose,

TABLE V

PRODUCT YIELD VS. SUCROSE CONCENTRATION USING STRAIN C42(3)E13 AT pH 6.2 UNDER NITROGEN

Product	Millimoles per 100 mM. invert sugar used				
	4% sucrose	5% sucrose	6% sucrose	8% sucrose	10% sucrose
2,3-Butanediol	54.7	54.8	59.7	65.2	71.2
Acetoin	4.4	3.7	0.9	2.4	3.0
Ethanol	66.7	66.2	64.6	60.6	40.7
Acetic acid	14.3	8.9	3.5	1.9	7.4
Formic acid	0.6	0	0.2	0.2	0.3
Succinic acid	0.2	0.3	0.1	0.2	0.2
Lactic acid	1.9	1.7	3.1	3.5	4.2
Total organic acids	17.0	10.9	6.9	5.8	12.1
Carbon dioxide	218	199	175	186	170
Hydrogen	88	86	63.7	64.5	68.3
Carbon assimilated			6.0		16.5
CO_2 obs./calc.	1.09	1.00	0.87	0.93	0.85
H_2 obs./calc.	0.88	0.86	0.64	0.65	0.68
$\frac{\text{Acetoin + diol}}{\text{Ethanol}}$ (by wt.)	1.6	1.7	1.8	2.2	3.6
Carbon recovery, %	103.9	98.4	94.9	98.9	99.0
O/R Index	1.11	1.02	0.94	0.96	0.92
Fermentation time, hr.	25	29	23	30	71
% invert sugar used	86.3	95.7	95.0	99.2	94.4
pH at beginning	6.20	6.20	6.20	6.20	6.20
Final pH	6.24	6.25	6.30	6.24	6.23

in which 99% of the sugar is dissimilated in 30 hr., producing 65 mM. of diol per 100 mM. of invert sugar fermented.

Reports by various authors indicate that the optimal sucrose concentration for diol production varies widely with the precise conditions under which the fermentation takes place. Fulmer, Christensen, and Kendall (12) found an optimal initial sucrose concentration for *Aerobacter* of 8%, while Freeman reported a maximum diol yield (87.4% of theoretical) with an initial sucrose concentration of 15% (11). In the former investigation 20 days was required to complete the fermentation, while in the latter complete sucrose conversion took place in 35 hr.

For comparison of the aerobic and anaerobic dissimilations of sucrose, a similar series using strain C42(3)E13 was performed using air in place of nitrogen. Under these conditions dissimilation is slower, and at concentrations of 8 and 10% is incomplete even after 148 hr. (Table VI). Total yields of diol plus acetoin are considerably augmented, and remain fairly constant at about 80 mM. per 100 mM. of sucrose fermented. Ethanol yields show the conspicuous depression known to accompany aeration (1, 37). The large increase in the diol–ethanol ratio, which reaches a maximum at 10%, is thus explained. In contrast to their formation under nitrogen, total organic acids

TABLE VI

AEROBIC DISSIMILATION OF SUCROSE IN VARYING CONCENTRATIONS AT pH 6.2 BY STRAIN C42(3)E13

Product	Millimoles per 100 mM. invert sugar used				
	4% sucrose	5% sucrose	6% sucrose	8% sucrose	10% sucrose
2,3-Butanediol	47.2	37.8	58.0	47.8	60.4
Acetoin	34.6	37.6	24.3	31.9	20.8
Acetoin + diol	81.8	75.4	82.3	79.7	81.2
Ethanol	11.6	23.7	20.2	17.6	13.8
Acetic acid	0.6	4.5	8.2	5.3	4.5
Formic acid	0.3	0.6	0.2	0	0
Succinic acid	0.2	0.2	0	0.2	0.2
Lactic acid	0.3	3.7	10.5	9.3	13.7
Total organic acids	1.5	9.0	18.9	14.8	18.4
Carbon dioxide	244	210	173	197	184
Hydrogen	1.0	2.0	1.0	1.0	0.8
CO_2 obs./calc.	1.22	1.05	0.86	0.98	0.92
H_2 obs./calc.	0.01	0.02	0.01	0.01	0.08
$\frac{\text{Acetoin + diol}}{\text{Ethanol}}$ (by wt.)	8.0	6.2	7.9	8.7	11.4
Carbon recovery, %	99.8	96.7	98.4	98.4	98.0
Fermentation time, hr.	59	104	71	148	148
% invert sugar used	98.8	89.5	96.5	83.4	78.5
pH at beginning	6.20	6.20	6.20	6.20	6.20
Final pH	6.31	6.21	6.21	6.20	6.27

increase under air with increasing sucrose concentration from 4 to 6%, and remain relatively constant at higher concentrations. Dissimilation of 6% sucrose becomes 98% complete in 71 hr., and produces 82% by weight of theoretical diol plus acetoin. The conspicuous and irregular differences in diol yield, along with the relative constancy of total diol plus acetoin, is further evidence of fluctuations in *Eh* (40). These data confirm the conclusion of Adams, based on the fermentation of wheat mashes under aerobic and anaerobic conditions (1).

The physical and chemical factors influencing each fermentation had been standardized as carefully as possible, but marked fluctuations are still apparent in carbon dioxide production and in ethanol and acid yields. These fluctuations have been ascribed to the inherent variability of the organism (2); the basis of part of this variability may reside in trace-element sensitivity (29).

The examples of irregular fermentation times provided in Tables V and VI are often observed in studies with *Bacillus polymyxa*. These apparently fortuitous variations are correlated with cessations of acid production recorded by the monitor (6). The duration of such 'rest periods' varied from several to many hours. Recovery invariably followed and dissimilation proceeded. Microscopic examination during a quiescent period revealed no visible abnor-

malities in the organism. Disappearance of a necessary growth factor and its subsequent resynthesis by the organism offers one of a number of possible explanations.

Discussion

The effects of pH on fermentation efficiency are slight from pH 6.0 to 6.4, although they become accentuated above and below this range (Tables III and IV). Increased fermentation times accompany reduction of the pH much below 6.0. Above pH 6.0 the increased production of total organic acids by both C3(2) and C42(3)E13 is correlated with a general decrease in carbon dioxide production. Moreover, diol yields begin to show a decrease above pH 6.4 and the increased acid production necessitates increased additions of alkali. Favorable yield of diol, low acid production, low alkali utilization, and favorable fermentation rate point to a maximum efficiency in the vicinity of pH 6.2. This corresponds closely with the recommended optimum of 6.0 in whole wheat mashes (2) and of pH 6.2 in glucose dissimilation (33). It also coincides with the optimum pH of 6.2 selected by Kendall for *Aerobacter* spp. (20) and this observation is supported by the evidence for a critical pH level of 6.3 for *A. indologenes*. It was demonstrated by Mickelson and Werkman that acetic acid is condensed to acetoin below pH 6.3, whereas above this level it accumulated as the free acid (28). If choice of the optimum pH is based on glycol yield, the data of Freeman point to an optimum from pH 5.0 to 5.5 for *A. aerogenes* 199. The initial rate of sucrose conversion was 25 to 30% greater at pH 6.0 to 6.5 than at 5.0 to 5.5, but the final glycol yield was 22% higher over the lower range (11). Analyses of end products in the present investigation provided no evidence that measurable changes in yields follow variations of $\pm$ 0.1 pH units. Statistical analysis of the results from a series of closely controlled runs would be necessary to determine the effects of such minor deviations.

The final pH values in Tables I to VI are relative. They were obtained by calibrating the monitor at a temperature of 33° $\pm$ 1° C. with a buffer standardized on the Sørensen scale at 18° C.* (7) and retaining the buffer to recalibrate a pH meter at room temperature at the end of the fermentation. The pH of the fermented liquor was *always* higher than that of the original setting, often by as much as one or more units. No critical analysis of the factors that may be responsible for this swing to alkalinity at the end of the fermentation period has been made. The difference in number of scale units between the original potentiometer setting and the final setting at re-balance divided by 295 gave the difference, x, in pH units from the original setting. The divisor (295) is based on a value of 0.059 v. per pH unit, and an exact calibration of 5.0 scale divisions per millivolt. Addition of the x value to the pH of the buffer and comparison of this total with the reading of the pH meter gave the 'final pH' values recorded in Tables I to VI. For 20 of the 23 fermentations the deviation is positive (average = + 0.08), i.e., the calcu-

**Clark and Lubs $NaOH.KH_2PO_4$ mixtures.*

lated pH is below that of the fermented liquor. This consistently positive deviation points to inaccuracy in one or more of the measurements. Differences between the standardization temperature of the buffer and that at which it is used in calibrating the monitor and the pH meter, changes in the liquid junction potential, disbalance in the electronic control circuits with resulting inequalities in calibration at different points on the scale, use of the RT/F factor at 25° C. (i.e., 8° C. below the fermentation temperature), and changes in electrode e.m.f. during the period of measurement are some of the factors that may have contributed to the positive deviation. An evaluation of these factors will be made in a subsequent study.

The adverse effects of sodium and potassium hydroxides shown in Table I may be correlated with changes in cell permeability induced by excessive addition of unbalanced sodium or potassium ion. The marked acceleration in fermentation rate with the addition of ammonium hydroxide may be linked with beneficial effects on nitrogen metabolism. Insufficient evidence is available to allow evaluation of these two separate effects of ammonia. Freeman (11) has noted that fermentation rates and diol formation by *Aerobacter aerogenes* were lower in media controlled by stepwise addition of sodium hydroxide than in the presence of calcium carbonate. The difference was attributed to the unavoidable width of the pH steps between successive additions of alkali. Although wide fluctuations in pH doubtless have considerable influence, the toxicity of excess sodium or potassium ion may be partly responsible for the low fermentation rates and diol yields. From a comparison of *B. polymyxa* fermentations of whole wheat mashes (*a*) buffered by calcium carbonate and (*b*) controlled by additions of ammonia, Adams and Leslie (2) selected the latter as preferable because of its inherent sterility, range of control, ease of addition in either the liquid or the gaseous state, and lower cost. To these advantages may be added a supply of available nitrogen and acceleration of the fermentation rate with automatic pH control.

All the products reported in the carbon balances have been characterized for this fermentation (9). Other compounds, e.g., malic acid and acetone, are known to occur in media fermented by certain strains of *B. polymyxa* (9, 36) and some evidence for the presence of small amounts of these substances was obtained. The formation of traces of acetone was revealed in about half of the fermentations by the oxime method (15) after applying corrections for acetoin (31). Traces of malic acid were disclosed by filter paper chromatography, and the application of Shupe's method (38) showed the possible presence of glycerol. These compounds were not present in amounts sufficient to affect the carbon balances and they have been omitted from the tables.

There is still no general agreement as to the mechanism by which acetoin arises in the degradation of carbohydrates by bacteria, although there is evidence that pyruvic acid and acetaldehyde may be the immediate precursors. (14, 39). The mode of ethanol formation is also obscure. A common

explanation of the effect of aerobic conditions on this fermentation has been that gaseous oxygen acts as a hydrogen acceptor, decreasing the reduction of acetaldehyde to ethanol and favoring its condensation to acetoin (1, 3). Rose pointed out the irrelevancy of this assumption (37) and Gale has recently suggested that *coli-aerogenes* organisms may produce ethanol directly from dihydroxyacetone phosphate (13). Most of the investigational evidence for this hypothesis has been obtained from members of the *coli-aerogenes* group and additional information must be collected before its extension to the carbohydrate metabolism of *B. polymyxa*.

Acknowledgments

The authors wish to express their indebtedness to Dr. A. C. Neish of the Prairie Regional Laboratory, to Mr. S. G. Harbottle of the Division of Applied Biology, and to Messrs. J. E. Breeze and J. Akeroyd of the Radio and Electrical Engineering Division for invaluable help and advice. They also wish to acknowledge with thanks the technical assistance of Messrs. D. R. Muirhead and J. L. Barnwell.

References

1. Adams, G. A. Can. J. Research, F, 24 : 1-11. 1946.
2. Adams, G. A. and Leslie, J. D. Can. J. Research, F, 24 : 12-28. 1946.
3. Adams, G. A. and Stanier, R. Y. Can. J. Research, B, 23 : 1-9. 1945.
4. Blackwood, A. C. and Ledingham, G. A. Can. J. Research, F, 25 : 180-191. 1947.
5. Breeze, J. E. Proc. Natl. Electron. Conf. 4 : 45-60. 1948.
6. Breeze, J. E. Natl. Research Council Can. Rept. No. ERA-166. 1949.
7. Clark, W. M. The determination of hydrogen ions. The Williams & Wilkins Company, Baltimore. 1928.
8. Dole, M. The glass electrode. John Wiley & Sons, Inc., New York. 1941.
9. Donker, H. J. L. Thesis. Meinema. Delft. Holland. 1926.
10. Fratkin, S. B. and Adams, G. A. Can. J. Research, F, 24 : 29-38. 1946.
11. Freeman, G. G. Biochem. J. 41 : 389-398. 1947.
12. Fulmer, E. I., Christensen, L. M., and Kendall, A. R. Ind. Eng. Chem. 25 : 798-800. 1933.
13. Gale, E. F. The chemical activities of bacteria. 2nd ed. Univ. Tutorial Press. London. 1948.
14. Gross, N. H. and Werkman, C. H. Arch. Biochem. 15 : 125-131. 1947.
15. Haughton, C. O. Ind. Eng. Chem. Anal. Ed. 9 : 167-168. 1937.
16. Katznelson, H. Can. J. Research, C, 22 : 235-240. 1944.
17. Katznelson, H. Can. J. Research, C, 24 : 99-108. 1946.
18. Katznelson, H. Can. J. Research, C, 25 : 129-136. 1947.
19. Katznelson, H. and Lochhead, A. G. Can. J. Research, C, 22 : 273-279. 1944.
20. Kendall, A. R. Iowa State Coll. J. Sci. 9 : 171-172. 1934.
21. Kratz, L. Glastechn. Ber. 20 : 305-315. 1942.
22. Ledingham, G. A. and Adams, G. A. Can. Chem. Process Inds. 28 : 742-746. 1944.
23. Ledingham, G. A., Adams, G. A., and Stanier, R. Y. Can. J. Research, F, 23 : 48-71. 1945.
24. Longsworth, L. G. and MacInnes, D. A. J. Bact. 29 : 595-607. 1935.
25. Longsworth, L. G. and MacInnes, D. A. J. Bact. 31 : 287-300. 1936.
26. Longsworth, L. G. and MacInnes, D. A. J. Bact. 32 : 567-585. 1936.
27. Mickelson, M. N. Iowa State Coll. J. Sci. 15 : 84-86. 1940.
28. Mickelson, M. N. and Werkman, C. H. J. Bact. 36 : 67-76. 1938.
29. Murphy, D. Private communication.
30. Neish, A. C. Can. J. Research, B, 23 : 10-16. 1945.
31. Neish, A. C. Natl. Research Council Can. Rept. No. 46-8-3. June 1946.
32. Neish, A. C. Can. J. Research, B, 27 : 6-20. 1949.
33. Neish, A. C. and Ledingham, G. A. Can. J. Research, B, 27 : 694-704. 1949.

34. OLSON, B. H. and JOHNSON, M. J. J. Bact. 55 : 209-222. 1948.
35. REYNOLDS, H. and WERKMAN, C. H. J. Bact. 33 : 603-614. 1937.
36. ROSE, D. Can. J. Research, F, 24 : 320-326. 1946.
37. ROSE, D. Can. J. Research, F, 25 : 273-279. 1947.
38. SHUPE, I. S. J. Assoc. Offic. Agr. Chemists, 26 : 249-256. 1943.
39. SILVERMAN, M. and WERKMAN, C. H. Proc. Soc. Exptl. Biol. Med. 43 : 777-778. 1940.
40. STAHLY, G. L. and WERKMAN, C. H. Biochem. J. 36 : 575-581. 1942.
41. STANIER, R. Y. and ADAMS, G. A. Biochem. J. 38 : 168-171. 1944.
42. TOMKINS, R. V., SCOTT, D. S., and SIMPSON, F. J. Can. J. Research, F, 26 : 497-502. 1948.
43. UNDERKOFLER, L. A., Guymon, J. F., RAYMAN, M. M., and FULMER, E. I. Iowa State Coll. J. Sci. 17 : 251-256. 1943.
44. UTTER, M. F., LIPMANN, F., and WERKMAN, C. H. J. Biol. Chem. 158 : 521-531. 1945.
45. VAN SLYKE, D. D. and FOLCH, J. J. Biol. Chem. 136 : 509-541. 1940.
46. WATSON, R. W. In preparation.
47. WHEAT, J. A., LESLIE, J. D., TOMKINS, R. V., MITTON, H. E., SCOTT, D. S., and LEDINGHAM, G. A. Can. J. Research, F, 26 : 469-496. 1948.

ON THE USE OF CRYSTAL CONTROLLED SYNCHRONOUS MOTORS FOR THE ACCURATE MEASUREMENT OF TIME[1]

By V. E. Hollinsworth

Abstract

Making use of the quartz frequency standard of the Dominion Observatory crystal clock an electronic frequency converter has been constructed that transforms the 50,000 cycle output of the crystal to a frequency of 60 cycles. Suitable amplification makes possible sufficient power for operating several standard 60 cycle motors and these are being used to drive printing and drum chronographs and for the operation of other types of recorders.

Introduction

There are many applications in scientific work for small synchronous motors capable of constant speeds of rotation. However, when such motors are operated from a commercial alternating current line, variations in frequency with resulting variations in motor speed often result in unsatisfactory performance. Particularly is this true in observatories and institutions concerned with the precise measurement and recording of time. For this reason a project was undertaken at the Dominion Observatory to develop a means of operating synchronous motors with a constancy of rotational speed corresponding to the performance of the best clocks. If accuracy of this order could be obtained, such motors could be used with suitable electrical contacts to operate clock dials, for use as interval timers or as drives for chronograph drums, printing chronographs, and other types of time recorders.

The principle adopted for the control of motor speed was to make use of the vibrational qualities of quartz crystals to provide an alternating current of constant frequency, and then by the use of frequency dividers followed by a stage of frequency multiplication and suitable amplification to produce 60 cycle power sufficient to operate a number of small synchronous motors.

Quartz has long been known to have piezoelectric properties (1), that is, it will transform a mechanical strain into an electrical charge and vice versa. A suitably cut quartz wafer (6) or bar may be sandwiched between two electrodes and made to vibrate mechanically at its resonant frequency by the application of alternating electrical pulses to the electrodes, and if the timing of the pulses is correct, the vibrations of the quartz will be maintained. The crystal may be coupled to an electrical circuit and in effect made to substitute for a coil and condenser resonant circuit (9). As the crystal will not vibrate at frequencies other than that at which it is resonant, the circuit that it controls will function only at that particular frequency.

[1] *Manuscript received in original form March 5, 1949, and, as revised, September 30, 1949. Vol. II, No. 6 Contributions from the Dominion Observatory.*
Published by permission of the Director, Mines, Forests, and Scientific Services Branch, Department of Mines and Resources, Ottawa, Canada.

If the temperature of the crystal is controlled within sufficiently narrow limits, in a suitable thermostat, the variations in the natural frequency of the crystal will be extremely small, so small in fact that such crystals have now become among the world's best timekeepers (2). Crystal controlled clocks have largely supplanted the pendulum type as master timekeepers in most observatories (3, 4, 5). Quartz crystals of suitable physical dimensions are also used to accurately maintain the broadcast frequencies of all radio stations.

The problem of crystal control of synchronous motors thus becomes that of obtaining a low frequency crystal and subdividing its natural period of vibration in steps, having a ratio of not greater than 10 to 1, until the desired output frequency is obtained. Precision quartz plates are usually cut to have a natural frequency of either 50 or 100 kc. (6). The first frequency divider would reduce this to 10 kc., the second to 1 kc., the third to 100 cycles, the fourth to 10 cycles, and this may then be multiplied six times to give 60 cycle current. As very little power is available from these frequency changing circuits, a heavy duty power amplifier as the final stage is required to supply sufficient voltage and current for the operation of a number of motors.

Let us now consider the practical construction of an Electronic Frequency Converter to operate on the principle outlined above. At the Dominion Observatory the availability of a crystal clock (General Radio Frequency Standard) with a 50 kc. quartz crystal and an output frequency of 1000 cycles provided an excellent base on which to build. It was necessary to construct only three units to convert this 1000 cycles to 60 cycles (7, 8) plus a power amplifier to deliver the desired voltage and current for motor operation.

The first unit reduced the frequency to 100 cycles, the second to 10 cycles, and the third increased it to 60 cycles. Other frequencies might have been chosen, viz., 1000 to 3000 cycles, 3000 to 300 cycles, and 300 to 60 cycles, but it was considered advantageous to have both 100 cycle and 10 cycle outputs available for other purposes.

An examination of the circuit diagram of the first unit, Fig. 1, will serve to illustrate the principles involved. The first tube is merely an amplifier to isolate the frequency determining circuit from the input line. The second and third tubes, with their associated condensers and resistors, generate recurring pulses at the rate of 100 per sec., since the values of condensers and resistors that will produce this frequency were selected.

The recurring feature is procured by feeding back some of the output voltage from the third tube to the grid of the second tube; this causes the circuit to oscillate. An adjustable resistor makes it possible to set the frequency of oscillation at the desired value. In practice this setting is just under 100 cycles so that the oscillator is just about ready to produce a pulse when every tenth pulse of the 1000 cycle control frequency arrives and initiates

it. This, then, is the method of control and may be carried on in the two following units to supply a final frequency of 60 cycles controlled by the 1000 cycle supply which, in turn, through intermediate stages is controlled by the 50 kc. crystal.

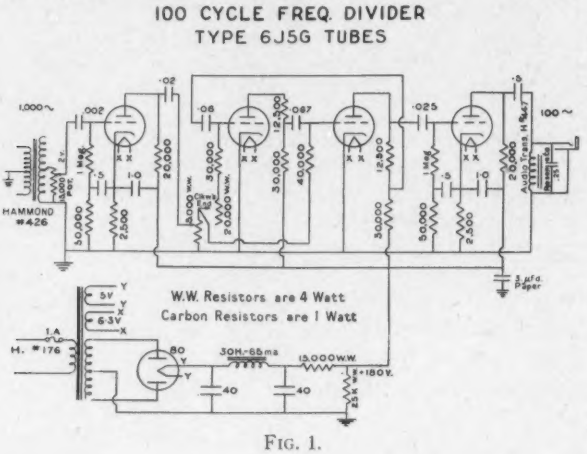


FIG. 1.

The fourth tube in the circuit is an isolating amplifier to prevent succeeding stages from interfering with the frequency determining network, and is also the place selected to install a resonant circuit, i.e., a transformer tuned to

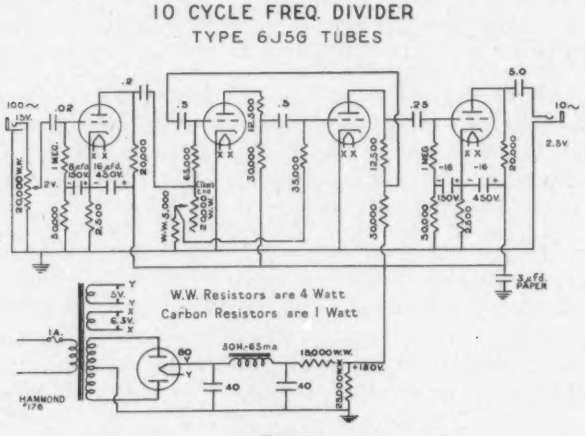


FIG. 2.

100 cycles by a condenser, to produce sine wave form from the "sawtooth" type generated by the oscillator. If this is not done the succeeding 10 cycle stage is likely to be triggered by one of several spurious peaks appearing on the ragged untuned wave.

The above description of the operation of the 100 cycle frequency divider applies generally to both the 10 cycle unit, Fig. 2, and the 60 cycle unit, Fig. 3. However, a few points in the 60 cycle unit warrant further description. As

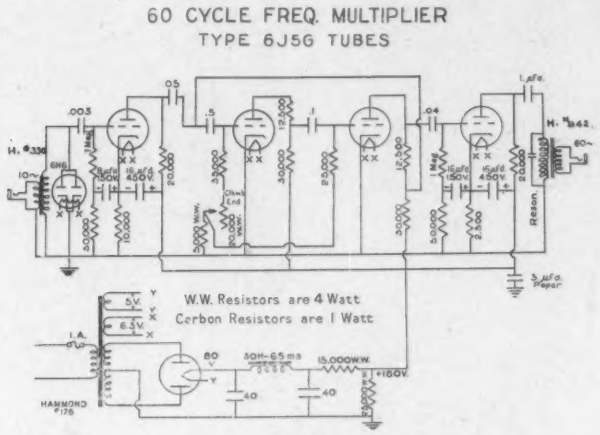


FIG. 3.

the 10 cycle pulse is normally much broader than a 60 cycle pulse, some means had to be found of narrowing the wave-form in order that a definite influence, or triggering action, should be exerted on a single wave, i.e., every

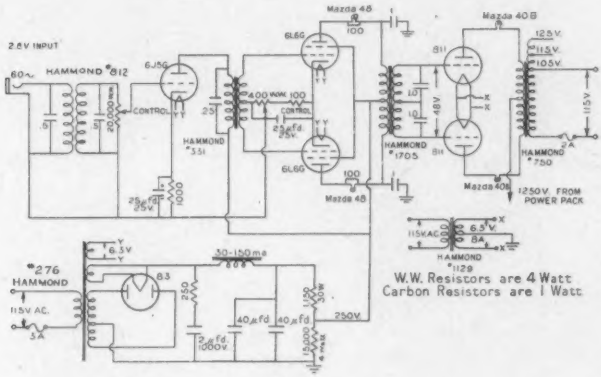


FIG. 4.

sixth wave of the 60 cycle unit, without disturbing the fifth or seventh waves. It was thought that this could be accomplished by using a high driving voltage on the grid of the first tube in the 60 cycle unit, and biasing the grid sufficiently

negative so that only the peaks of the positive swing would be effective in changing the plate current of this tube. As the peak is much narrower than the whole wave this method would appear to be satisfactory. This was found to be so when the input circuit, Fig. 3, was constructed as shown. An audio transformer with a 1 : 3 turn ratio was used to boost the voltage and a 10,000 ohm cathode resistor supplied sufficient bias. The unwanted negative portion of the voltage wave was shorted out by suitably connecting a half wave rectifier across the transformer secondary.

In other respects the 60 cycle unit is similar to the 100 cycle unit described above.

AMPLIFIER POWER SUPPLY

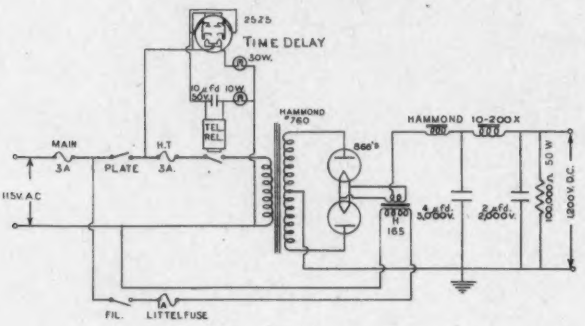


FIG. 5.

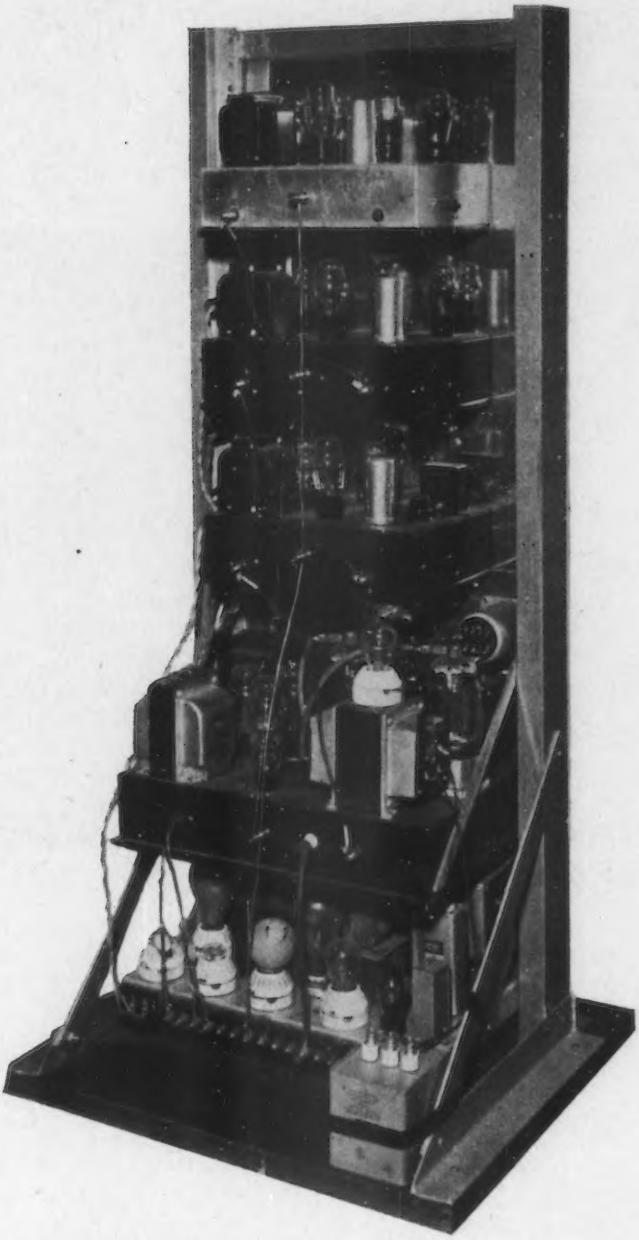
A heavy duty amplifier, Fig. 4, was constructed to build up the output of the 60 cycle unit to supply sufficient power both for the operation of synchronous motors and for providing stability so that the addition or removal of several motors would not seriously alter the voltage. The one constructed for use at the Dominion Observatory will deliver up to 150 w. at 115 v. with sine wave form. This power is sufficient to operate quite a number of small motors drawing from 2 to 10 w. each.

Type 811 tubes were used, as these are capable of delivering the required power and are available cheaply as war surplus.

A conventional class "B" circuit was employed, using push-pull 6L6 tubes as drivers, which were, in turn, controlled by a single 6J5 input tube. The input circuit was resonated at 60 cycles to ensure that the input, and hence the output, would be of sine wave form.

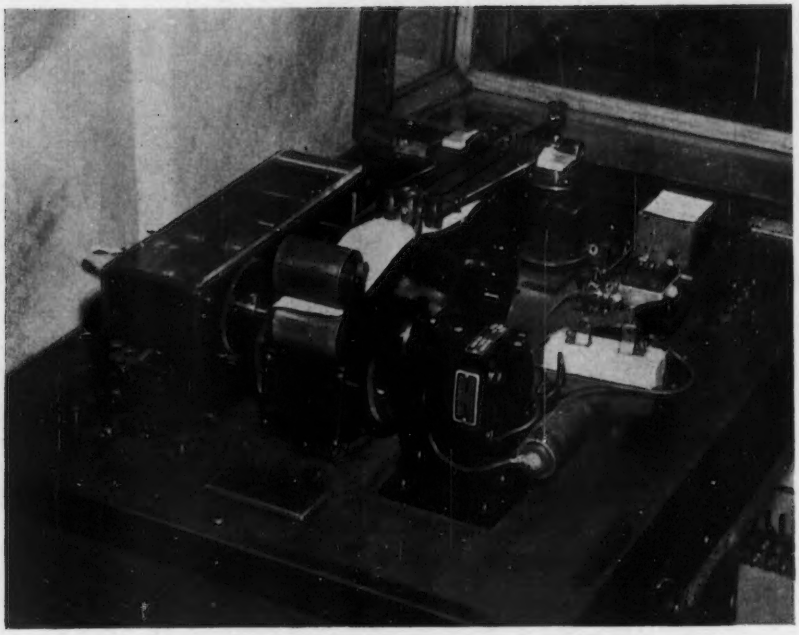
A heavy duty power supply, Fig. 5, was constructed on a separate chassis to supply the plate voltage for the Type 811 tubes. These require 1200 v. at 200 ma. for full output, so components capable of delivering this power were used. As our present loading of 40 w. represents less than 30% of full load capacity, the equipment runs cool and has a large factor of safety. As

PLATE I



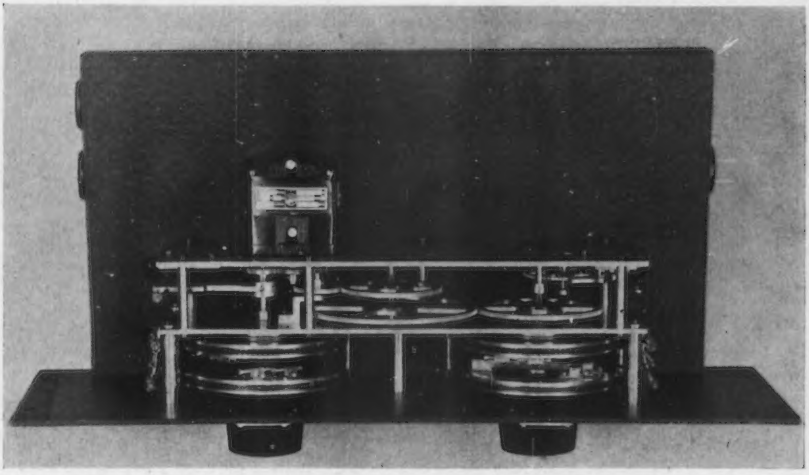
Rear view of Dominion Observatory electronic frequency converter, etc.

PLATE II



Showing the application of a synchronous motor to drive the type wheels of a printing chronograph.

PLATE III



A synchronous motor driven time converter to derive sidereal time from mean time.

many as 10 motors could be operated continuously and still leave a satisfactory operating margin.

Good filtering of the rectified a. c. is required to avoid locking-in of the 60 cycle controlled frequency with the a-c. line frequency.

All components used throughout were underrated so that the resulting large safety factor would minimize break downs.

Many uses are being found for this crystal controlled source of power. Both high and low speed recording chronograph drums are being driven by synchronous motors. The rotating type wheels of the printing chronograph, Plate II, are also being operated this way with considerable improvement over the governor method. Small synchronous clock motors equipped with contacts are being used to give seconds impulses and a gear train Time Converter, Plate III, has been constructed to produce sidereal time from mean time when synchronously driven.

To measure the accuracy, as a timekeeper, of a motor controlled by the above power supply, a second's contact was actuated by the rotor of a Bodine motor (60 r.p.m.). Comparisons were made with the original crystal clock, and the variation between the two was of the order $\pm 1 \times 10^{-3}$ sec. This was the limit of measurement and very likely the variation is considerably less than this quantity.

All units are rack mounted as illustrated on Plate I. This affords economy of space and enhances the appearance.

Early causes of "pull out" have been analyzed and corrected, and during the past year the operation of this equipment has been very satisfactory.

References

1. CADY, W. G. Piezoelectricity. McGraw-Hill Book Company, Inc., New York. 1946.
2. HEATON, V. E. Instruments, 20 (7) : 618. 1947.
3. HOPE-JONES, F. Horological J. October, 1943.
4. MARRISON, W. A. Horological J. Vol. 90, Nos. 1076-1081. 1948.
5. MARRISON, W. A. Proc. Natl. Acad. Sci. U.S. 16 : 496-507. 1930.
6. MASON, W. P. Proc. Inst. Radio Engrs. 28 (No. 5) : 220-223. 1940.
7. MILLER, R. L. Proc. Inst. Radio Engrs. 27 (No. 7) : 446-457. 1939.
8. VAN DER POL, B. and VAN DER MARK, J. Nature, 120 : 363-364. 1927.
9. VAN DYKE, K. S. Phy. Rev. 25 : 895. 1925.

FACTORS AFFECTING THE DETERMINATION OF MOISTURE IN HARD RED SPRING WHEAT BY COMMERCIAL ELECTRICAL METERS[1]

By A. E. Paull[2] and V. Martens[3]

Abstract

Statistical analysis of data obtained with eight commercial electrical moisture meters and 159 samples of Hard Red Spring wheat shows that variations in bushel weight and kernel weight have an effect on the moisture readings but variations in protein and ash content do not. An appreciable amount (from 10 to 30%) of the variation in the G.R.L., Steinlite, Marconi, and Mullard readings is attributable to variations in bushel weight alone; and in the Patterson and Tag–Dielectric readings to bushel weight and kernel weight jointly. The Tag–Heppenstall and Universal meters are not influenced appreciably by the factors investigated.

Introduction

A recent paper from this Laboratory (4) presented results of a comparative study of 10 electrical moisture meters based on observations on 159 samples of hard red spring wheat. All machines measured the electrical property of the samples, either resistance or capacitance, with good precision, but for some machines the correlation between the electrical property and moisture content was considerably better than for others. Electrical properties of a grain sample are dependent also on factors other than moisture content, and these additional factors have a greater influence with some meters than with others. The present paper shows that the average size and shape of the wheat kernels, as estimated by bushel weights or 1000-kernel weights, affect meter readings, but that the readings are not affected by the protein or ash content of the samples.

Materials and Methods

Data for eight meters, Tag–Heppenstall, Universal, Tag–Dielectric, G.R.L., Patterson, Steinlite, Mullard, and Marconi, have been examined. An earlier paper (4) described methods used for determining the moisture contents of 159 wheat samples by the split vacuum oven method and with the electrical meters. Other measurements were made by standard methods. Ranges for the series of samples were: moisture, 11.7 to 17.6%; bushel weight, 55 to 65 lb.; 1000-kernel weight, 17.8 to 35.1 gm.; protein content, 12.4 to 19.2%; and ash content, 1.27 to 1.98%. Levels of these properties were distributed randomly over the respective ranges.

[1] *Manuscript received July 29, 1949.*
Paper No. 102 of the Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg, Manitoba, and No. 276 of the Associate Committee on Grain Research.
[2] *Biometrician.*
[3] *Senior Technician.*

Multiple regression methods were used to study the possible effects of these properties on meter estimates of moisture content, and tests of significance were applied to determine whether the effects observed were likely to have arisen other than by chance. Those factors for which a real effect was demonstrated were then examined to determine by how much the error of estimating moisture would be reduced if the effects of the factor were eliminated.

Results

Bushel weight exhibits an influence on moisture readings for all the meters tested, and kernel weight exhibits an influence on all except the Mullard, although in both cases the effects for the Tag–Heppenstall, Universal, and Tag–Dielectric meters are so small as to be of little practical importance. Neither protein nor ash content gives evidence of influencing the moisture readings of any of the meters.

TABLE I

STANDARD ERRORS OF ESTIMATE IN MOISTURE READING UNITS AFTER ADJUSTING FOR MOISTURE CONTENT, BUSHEL WEIGHT, AND KERNEL WEIGHT

	Total error of estimate of moisture	Errors of estimate after adjusting for:		
		Moisture and bushel weight	Moisture and kernel weight	Moisture, bushel weight, and kernel weight
Tag–Heppenstall	0.23	0.23	0.22	0.21
Universal	0.27	0.27	0.26	0.25
Tag–Dielectric	0.34	0.34	0.33	0.31
G.R.L.	0.35	0.25	0.33	0.24
Patterson	0.39	0.38	0.39	0.34
Steinlite	0.41	0.30	0.38	0.30
Mullard	0.45	0.40	0.42	0.40
Marconi	0.53	0.39	0.50	0.38

A more detailed account of the relative effect of bushel weight and kernel weight is given in Table I. Standard errors of estimate are presented for the eight meters when no account is taken of kernel characteristics, and also when adjustments are made for bushel weight and kernel weight measurements both separately and jointly. For example, the entries for the G.R.L. meter show that, by the usual procedure, two-thirds of the moisture estimates given by the meter will lie within ± 0.35 of their true value. If, however, over the same series of samples, allowance is made for the variations in bushel weight, then two-thirds of the estimates will lie within ± 0.25 of their true values. Making allowance for kernel weight instead results in a standard error of ± 0.33, while adjusting for both bushel weight and kernel weight results in a standard error of ± 0.24. Generally, adjusting for bushel weight alone yields estimates at least as good as those obtained by adjusting for kernel weight alone; and except on the Mullard machine, adjusting for both factors

jointly results in significantly better estimates than adjusting for only one of these factors, although the improvement in this case is sufficiently large to be of practical importance only in the Patterson and Tag–Dielectric meters.

Discussion

No evidence was obtained that the nonaqueous constituents of the kernels affect electrical properties. Though the series of samples represented wide ranges of protein and ash contents, neither of these constituents, nor any that may be closely associated with them (e.g., starch), appear to have an appreciable effect on the resistance or capacitance of the wheat. Neitzert (5), who also gives an extensive review of European and American studies of moisture meters, shows a relation between specific resistance and ash content for three samples of flour. An increase of 1% in ash content appears to result in an increase of about 1% in the estimate of moisture. No similar relation was established in this laboratory for wheat, though there was some indication of a minor effect.

Among other factors that affect the estimates, temperature is of marked importance (1, 4), and this fact is taken into account by the manufacturers of all meters. Abnormal moisture distribution has been shown to have a marked effect on the electrical estimation of moisture content. Hartshorn and Mounfield (3) observed that artificially damped wheat tends to give unduly high readings. Abnormalities in the moisture distribution doubtless account also for the widely experienced difficulty in obtaining accurate meter estimates of moisture content in freshly harvested wheat and in wheat that has been rapidly dried.

There is a high correlation between bushel weight and kernel weight, so that it is difficult to conceive of the effects of these factors separately. Taken jointly, they may be considered as a good measure of kernel size and weight. It thus appears that these two properties affect moisture readings in all meters. Adjustments for bushel and kernel weights reduced the error of estimate least for the resistance type meters, Tag–Heppenstall and Universal, in which differences in size and shape of kernels are offset by partial crushing of the grain. The largest reductions occur in the Marconi, Steinlite, and G.R.L. meters.

Differences in size and shape of kernels can influence electrical readings in several ways. Variations in these properties result in variations in the closeness of packing in the dielectric type meters. This in turn yields differences in the grain/air ratio with resulting differences in the specific inductive capacity of the mixture. Yevstingneyev (6) dealt extensively with this matter in his study of measurement of the dielectric constant in flour. His paper also reviews earlier work dating back to 1894.

A further consequence of variations in kernel characteristics is the differences in weight, from sample to sample, when a fixed volume of grain is used; or

the differences in volume when a fixed weight of grain is used. For example, the Marconi meter employs a fixed volume of grain, so that it estimates essentially the moisture percentage by volume. Percentages by volume will differ from percentages by weight, over a series of samples, if there are differences in bushel weight. Accordingly, it was to be expected that the partial regression coefficient for bushel weight would be larger for the Marconi meter than for any of the others. A change of 1 lb. in bushel weight resulted in a change of 0.23% in the Marconi moisture reading; the next largest coefficient was 0.18% for the Steinlite meter, whereas the smallest coefficient for capacity type meters was 0.09% for the Tag–Dielectric.

In meters using a constant weight of grain, variations in size and shape of kernels create differences in the volume of the test sample. The load line of the condenser may thus vary from sample to sample, and resulting changes in end effect in the condenser will contribute to the error of estimate. This difficulty has been emphasized by Groves and King (2). It appears that it might be largely overcome by compression of a constant weight of grain to a constant volume.

References

1. Cook, W. H., Hopkins, J. W., and Geddes, W. F. Can. J. Research, 11 : 409-447. 1934.
2. Groves, L. G. and King, J. J. Soc. Chem. Ind. (London), 63 : 320-324. 1945.
3. Hartshorn, L. and Mounfield, J. D. Milling, 102 : 24-27. 1944.
4. Hlynka, I., Martens, V., and Anderson, J. A. Can. J. Research, F, 27 : 382-397. 1949.
5. Neitzert, Kurt. Getreide, Mehl und Brot. March. 1948.
6. Yevstingneyev, V. B. Cereal Chem. 16 : 336-353. 1939.

ESTIMATION OF LIPASE IN DAIRY PRODUCTS

I. AN EXTRACTION-TITRATION METHOD FOR THE ESTIMATION OF MILK LIPASE[1]

By L. M. Smith[2], D. J. Lubert[3], and H.R. Thornton[4]

Abstract

A procedure has been outlined for the estimation of milk lipase. The method is based on the titration of an ether extract of butyric acid derived from the enzymatic hydrolysis of tributyrin under standardized conditions. Some of the fundamental factors involved in the procedure have been discussed. It is suggested that modifications of the method may be used to study lipase activity in other dairy products and in cultures of micro-organisms.

Introduction

Early methods for the determination of milk lipase have been reviewed by Rice and Markley (5) and Hileman and Courtney (2) and will not be discussed in this paper. The presence of free fatty acids indicating lipase activity in dairy products may be detected by organoleptic means, lowered surface tension of milk, or titration against a standard alkali. The first two methods are susceptible to interfering factors and are not quantitative. The third method has been used by various investigators including Mattick and Kay (3). Peterson *et al.* (4) eliminated the time-consuming steam distillation required in the Mattick and Kay method. Dunkley (1) modified the Peterson *et al.* technique by using an ether extraction to avoid titrating the liberated butyric acid in the presence of milk salts, proteins, and the barbiturate buffer. The present paper deals with a further modification of the method suggested by Dunkley.

The Method

Reagents

Borate buffer: 6.23 gm. boric acid and 50 ml. *N* sodium hydroxide per liter of solution

Titration mixture: 50 ml. 0.1% alcoholic cresol red in 1 liter 95% alcohol and 1 liter distilled water. Adjust mixture to pH 7.4 with 0.05 *N* sodium hydroxide.

Tributyrin (Eastman)

Molar phosphoric acid

Ethyl ether

Standard 0.025 *N* sodium hydroxide.

[1] *Manuscript received July 26, 1949.*
Contribution from the Department of Dairying, University of Alberta, Edmonton, Alberta.
The data contained herein were taken from theses submitted to the University of Alberta by the two senior authors in partial fulfillment of the requirements for the degree of Master of Science. The study was supported by a grant from the Committee on Agricultural Research Grants of the University of Alberta.

[2] *Lecturer in Dairying.*
[3] *Sessional Instructor in Dairying.*
[4] *Professor of Dairying.*

Procedure

The sample of milk to be tested is centrifuged and 2 ml. of the skim milk is pipetted into a test tube containing 5 ml. of the borate buffer. The mixture is adjusted, if necessary, to pH 8.8 using 0.1 *N* sodium hydroxide or 0.1 *M* phosphoric acid. The buffered milk is brought to 37° C. in a water bath at that temperature within four minutes, 0.6 ml. of tributyrin is added and emulsified by vigorous shaking of the stoppered tube for five seconds. The mixture is then returned to the water bath and left undisturbed during the reaction period.*

At the end of the reaction period (30 min. after the addition of tributyrin) 5 ml. of molar phosphoric acid is added to stop hydrolysis and the test tube is inverted twice before transferring to a 20° C. water bath. After five minutes the mixture is shaken vigorously with 10 ml. of ethyl ether for five seconds. The test tube is returned to the water bath for another five minutes to allow the ether layer to separate and then the rubber stopper is loosened. After a further three minutes a 5 ml. aliquot of the ether layer is pipetted into a 50 ml. beaker containing 10 ml. of the titration mixture.

The solution is titrated with standard 0.025 *N* sodium hydroxide to a faint orange color and the number of milliliters required is designated as the "Titer". The blank determination is made in a similar manner except that the reaction period is omitted. Hydrolysis of the tributyrin is considered to be proportional to the concentration of lipase and is expressed as the difference between the sample and the blank titers.

The blank determination is affected by the presence of any free butyric acid in the tributyrin as well as by the presence of any organic acids in the milk sample. The blank titers of fresh milks are quite constant for a particular lot of tributyrin. For routine analyses of such milks this blank value can be used as a standard correction and only one titration carried out for each sample. However, this is not possible when dealing with milks that have undergone lipolysis or bacterial decomposition. In these samples the free fatty acids or such organic acids as acetic and lactic increase the value of the blank.

Experimental Techniques

The above procedure was followed in principle in the experimental work on the fundamental factors involved in the method. Buffers other than the standard differed only in the sodium hydroxide concentration. To keep the total volume for extraction reasonably uniform, 3.5 ml. of 1.5 *M* phosphoric acid was used in some of the experiments. All pH values were determined with a Beckman glass electrode pH meter. The milk samples were obtained from the University of Alberta herd and were held at 4° C. for periods from 2 to 48 hr.

* *The biochemical literature frequently but erroneously refers to this as an incubation period. As a true incubation period is an integral part of this procedure when adapted to the estimation of bacterial lipases, the time during which hydrolysis is permitted will be spoken of herein as the reaction period.*

Experimental

Titration of the Ether Extract

The proposed method involves the extraction and titration of butyric acid in the presence of boric and phosphoric acids. Solutions of these acids were prepared in the approximate concentrations in which they would be present in the reaction mixture at the time it is extracted with ether. Ten milliliters of each solution was extracted as in the method and potentiometric titrations were carried out. The resulting curves are shown in Fig. 1.

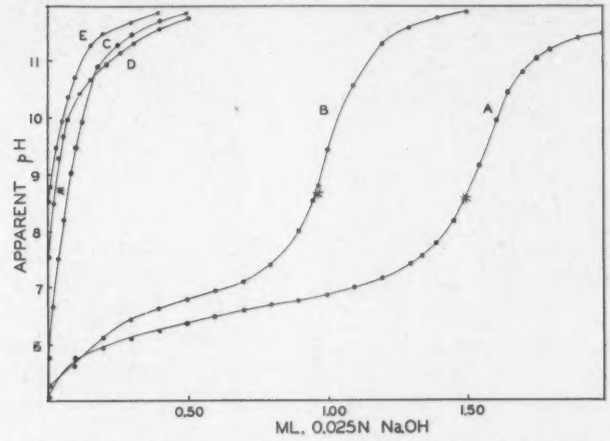


FIG. 1. *Potentiometric titration curves of ether extracts of: A, 0.008 M butyric acid; B, 0.005 M butyric acid; C, 0.5 M phosphoric acid; D, 0.05 M boric acid; E, distilled water. *Cresol red end point.*

It is apparent from these curves that butyric acid is easily extracted and that cresol red provides a suitable end point. The presence of low concentrations of boric and phosphoric acids does not interfere with the titration of the butyric acid.

Extraction of Free Butyric Acid

Increasing amounts of 0.05 *N* butyric acid were added to test tubes containing 7 ml. of buffered pasteurized skim milk after the addition and emulsification of the tributyrin. Following acidification with 3.5 ml. of 1.5 *M* phosphoric acid, the determinations were completed as outlined under "Method".

Fig. 2 shows that there was a direct relation between the concentration of free butyric acid in the sample and the amount extracted by the ether. Approximately 90% of the added butyric acid was recovered.

Experiments showed that minor changes in the extraction temperature and procedure, tributyrin concentration, buffer composition, and phosphoric acid concentration had little effect on the extraction of butyric acid.

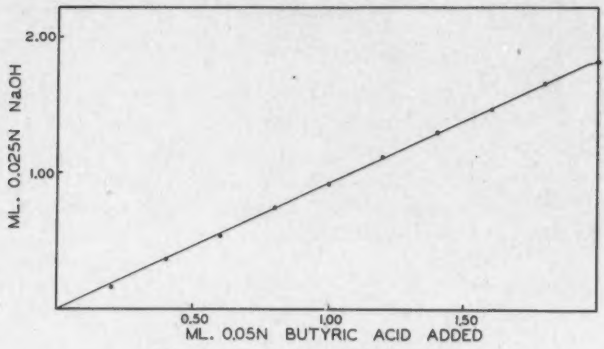


FIG. 2. *Extraction of butyric acid.*

SOME FACTORS AFFECTING THE METHOD

Tributyrin Concentration

Experiments were carried out in which the amount of substrate added was varied from 0.00 to 1.20 ml. The determinations were completed in the usual manner except that 3.5 ml. of 1.5 *M* phosphoric acid was used for acidification.

The data obtained for six milks are shown in Fig. 3. Hydrolysis close to the maximum was obtained when 0.60 ml. was used, except in the experiment on the highly active milk *A*. With higher concentrations of tributyrin less

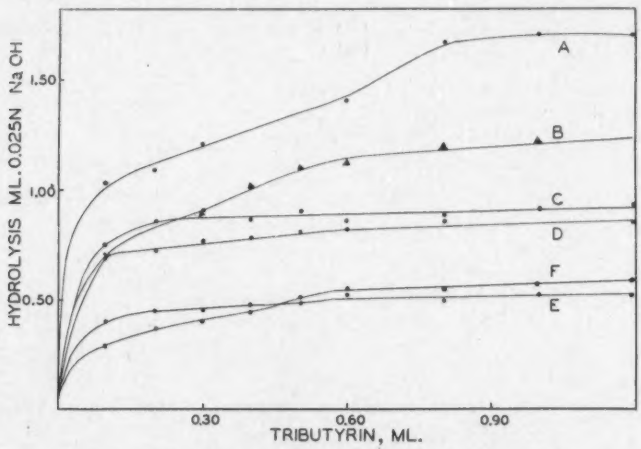


FIG. 3. *Substrate concentration and hydrolysis (six milks).*

ether separates as a clear layer and greater care is necessary in pipetting the 5 ml. aliquot for titration. The great majority of the determinations made on the individual milks of cows in the University of Alberta herd have given hydrolysis values under 1.25 ml. Therefore, 0.6 ml. of tributyrin is used for

routine assays. For milks of exceptionally strong lipase activity it may be convenient to halve the sample size or the reaction period.

Initial pH

A series of buffers was prepared that gave a suitable range of pH when 0.6 ml. of tributyrin had been emulsified in 7 ml. of buffered milk. The pH values of the mixtures were determined at 30° C. at the start of the reaction period and lipase assays were made on a duplicate series. Fig. 4 shows the

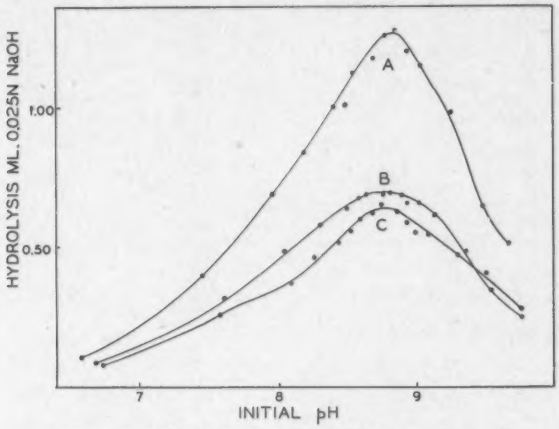


FIG. 4. *Initial pH and hydrolysis (three milks).*

curves obtained for three milks. While maximum hydrolysis occurred at approximately pH 8.75, this is not necessarily the optimum pH for milk lipase, because the hydrogen ion concentration does not remain constant throughout the reaction period. To study the buffer capacity of the borate buffer used in this procedure quadruplicate test tubes, each containing 7 ml. of buffered milk, were prepared. One tube was used for a pH determination at the beginning and one at the end of the reaction period. Lipase estimations were carried out on the remaining two tubes, one of them serving as a blank. The pH dropped as much as 0.40 unit in a highly lipolytic milk, as reported in Table I. It was found that no significant saponification occurred during the reaction period.

TABLE I

BUFFER CAPACITY OF SODIUM BORATE BUFFER

Milk	Initial pH 30° C.	Final pH 30° C.	pH difference	Hydrolysis
1	8.65	8.53	0.12	0.31
2	8.69	8.55	0.14	0.46
3	8.64	8.42	0.22	0.86
4	8.64	8.43	0.21	0.79
5	8.70	8.56	0.14	0.61
6	8.78	8.59	0.19	0.60
7	8.75	8.35	0.40	1.37
8	8.74	8.44	0.30	1.08

Temperature

Fig. 5 shows that in a 30 min. reaction period maximum hydrolysis was at 37° C. and this temperature was chosen for the method.

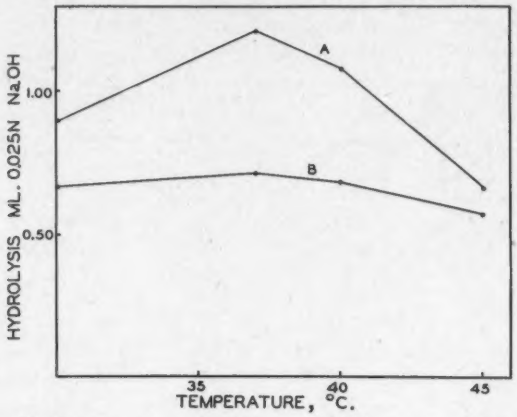


Fig. 5. *Reaction temperature and hydrolysis (two milks).*

Length of Reaction Period

Experiments in which the reaction time was varied from 0 to 90 min. showed that for the first 20 min. the rate of hydrolysis was constant and then started to decrease, presumably because of heat-inactivation. Fig. 6

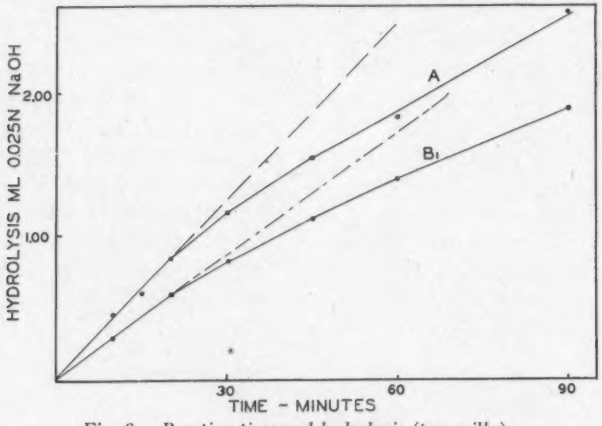


Fig. 6. *Reaction time and hydrolysis (two milks).*

shows the curves obtained in two representative experiments with different milks. Thirty minutes was chosen as the reaction time in order to obtain reasonable hydrolysis and yet avoid marked inactivation of the enzyme.

Enzyme Concentration

Varying amounts of suitable buffers, skim milk, and distilled water were added to test tubes to make a total volume of 7 ml. per tube and, after the

addition of 0.6 ml. of tributyrin, to give pH values of 8.75±0.05. Fig. 7 shows hydrolysis values in two representative trials. It is apparent that in these experiments hydrolysis of the substrate was proportional to the concentration of the enzyme.

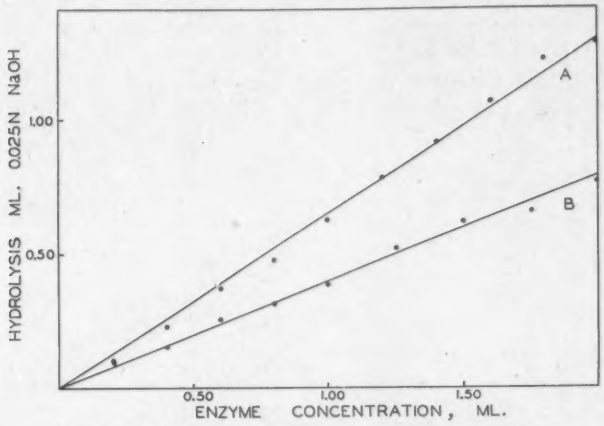


FIG. 7. *Enzyme concentration and hydrolysis (two milks).*

Reproducibility

In general, while duplicate determinations usually check to within ±0.02 ml. and variability among replicates tends to increase with increasing titer, nevertheless, there are some exceptions, as illustrated in Table II.

TABLE II

REPRODUCIBILITY OF LIPASE DETERMINATIONS

Milk	Titer	Blank	Hydrolysis	Total variation
1	1.07	0.22	0.85	0.02
	1.07		0.85	
	1.06		0.84	
	1.05		0.83	
2	1.10	0.23	0.87	0.07
	1.10		0.87	
	1.17		0.94	
	1.11		0.88	
3	1.74	0.21	1.53	0.06
	1.80		1.59	
	1.75		1.54	
	1.78		1.57	
4	2.14	0.24	1.90	0.04
	2.18		1.94	
	2.15		1.91	
	2.16		1.92	

Individual Milks

In the course of this investigation many assays were made on the milks of cows in the University herd, which included Jersey and Holstein breeds. Table III gives representative data for 10 such milks that had been held at 4° C. for approximately two hours after milking. No explanation to account for the variation in the lipase activities of these milks is advanced at this time. Further studies on the possible correlation between various management factors and lipase content of the individual milks are required. Factors such as storage conditions affecting the activity of milk lipase should also be investigated.

TABLE III

Lipase estimations on individual milks

Milk	1	2	3	4	5	6	7	8	9	10
Hydrolysis	0.38	0.48	1.16	0.80	1.35	0.51	1.08	0.56	0.56	0.57

Discussion

There is the possibility that milk lipase, which hydrolyzes milk fat, and the enzyme that splits tributyrin may not be identical. Therefore, some authors use the designation tributyrinase for the agent responsible for the latter reaction. The present authors prefer the term lipase until evidence substantiates the double enzyme theory.

The method described permits a comparatively simple and reasonably quantitative estimation of lipase in cow's milk. The lipolytic activity of milk is ordinarily not strong compared to that of some other biological fluids. The short reaction period entailed in this method may be found to be disadvantageous when the lipolytic activity is unusually weak. However, it is believed that the method may serve as a basis for the determination of lipase and the study of lipolysis in dairy products and microbial cultures.

References

1. Dunkley, W. L. Department of Dairying, University of Alberta. Unpublished data. 1946.
2. Hileman, J. L. and Courtney, E. J. Dairy Sci. 18 : 247-257. 1935.
3. Mattick, E. C. V. and Kay, H. D. J. Dairy Research, 9 : 58-71. 1938.
4. Peterson, M. H., Johnson, M. J., and Price, W. V. J. Dairy Sci. 26 : 233-240. 1943.
5. Rice F. E. and Markley, A. L. J. Dairy Sci. 5 : 64-82. 1922.

ESTIMATION OF LIPASE IN DAIRY PRODUCTS

II. AN EXTRACTION-TITRATION METHOD FOR THE ESTIMATION OF BACTERIAL LIPASE[1]

By D. J. Lubert,[2] L. M. Smith,[3] and H. R. Thornton[4]

Abstract

A method of estimating bacterial lipase is presented that is believed to be more nearly quantitative than any other at present available. The method is based on the titration of acids in an ether extract of a skim milk culture following a 30 min. period of lipase activity at 37° C. It is shown that ether-soluble acids carried into the reaction medium do not interfere with the measurements and that ether-soluble acids are not produced from protein or lactose during the test. The method is applicable over a sufficiently wide pH range to make it generally adaptable in bacteriology.

Introduction

Plating methods of demonstrating bacterially induced lipolysis have limited quantitative value and may be applied only within a restricted pH range. The advantage of their long incubation periods may be lost by the potentialities thus offered for indirect and interfering reactions (4).

The quantitative methods that have been proposed involve alkali titration of an inoculated and incubated emulsion of a fat in a liquid medium (5, 7, 10). These methods have certain disadvantages, including cumbrousness, long incubation periods with possible interfering reactions, titration in the presence of proteins, or obscure end points (8).

The method for estimating milk lipase that was described in the first paper of the present series (9) was suitably modified to measure bacterial lipases. This technique eliminates some of the disadvantages mentioned above and, as will be shown, is flexible over a wide range of conditions.

The Method

Reagents

Borate buffer: 6.23 gm. boric acid and 42 ml. *N* sodium hydroxide per liter of solution

Titration mixture: 50 ml. 0.1 alcoholic cresol red in 1 liter 95% alcohol and 1 liter distilled water. Adjust mixture to pH 7.4 with 0.05 *N* sodium hydroxide.

[1] *Manuscript received July 26, 1949.*
Contribution from the Department of Dairying, University of Alberta, Edmonton, Alberta.
The data contained herein were taken from theses submitted to the University of Alberta by the two senior authors in partial fulfillment of the requirements for the degree of Master of Science. The study was supported by a grant from the Committee on Agricultural Research Grants of the University of Alberta.

[2] *Sessional Instructor in Dairying.*
[3] *Lecturer in Dairying.*
[4] *Professor of Dairying.*

Standard 0.02 *N* sodium hydroxide
Tributyrin
Molar phosphoric acid
Ethyl ether.

Procedure

Ten milliliters of sterile skim milk is inoculated with a loop transfer from an actively growing slant culture of the test organism and incubated for 24 to 48 hr. at the organism's optimum growth temperature.

Two milliliters of this skim milk culture is pipetted into 5 ml. of the borate buffer in a test tube and adjusted, if necessary, to pH 8.5 with normal sodium hydroxide or molar phosphoric acid. This buffered culture is brought to the temperature of a 37° C. water bath within four minutes and 0.2 ml. tributyrin is added and emulsified by vigorous shaking of the stoppered tube for five seconds. The mixture is not further disturbed in the water bath during the remainder of the reaction period.

At the end of the reaction period (30 min. after the addition of the tributyrin) 5 ml. of molar phosphoric acid is added to stop hydrolysis and permit subsequent extraction of the liberated acid. The whole is mixed by inverting twice, brought to the temperature of a 20° C. water bath and shaken vigorously for five seconds with 10 ml. of ethyl ether. Ether layer separation and small water droplet settling are effected in five minutes in the undisturbed tube and then the rubber stopper is loosened. After another three minutes a 5 ml. aliquot of the ether layer is pipetted into a 50 ml. Erlenmeyer or beaker containing 10 ml. of the titration mixture.

Titration is usually with 0.02 *N* sodium hydroxide. It is probable that this concentration will be found generally satisfactory for the assay of bacterial lipase. As titer increases above approximately 1.5 to 2.0, sharper end points are obtained with increasing concentration of sodium hydroxide. By calculation such a titer value is brought to the equivalent 0.02 *N* sodium hydroxide value.

Titer is defined as the average number of milliliters of 0.02 *N* sodium hydroxide required in duplicate titrations. A blank determination is made in a similar manner except that the reaction period is omitted. Lipolysis is expressed as the difference between the sample and blank titers.

Experimental Techniques

In the experimental work the above procedure was followed in almost all cases but occasional variation was desirable. In a few experiments lipolysis was stopped by the addition of 3.5 ml. of 1.5 *M* phosphoric acid in the interests of volume uniformity for extraction.

Buffers other than the standard buffer were 0.1 *M* with respect to boric acid but contained varying amounts of normal sodium hydroxide or molar phosphoric acid.

In the accompanying tables the titer before and the titer after refer to the titers at the start and end of the reaction period.

The main test organism was a strain of *Pseudomonas fluorescens* growing well at 25° C. and poorly at 37° C. *Escherichia coli* was used in two experiments and *Streptococcus lactis* in another.

Plate counts were made according to Standard Methods for the Examination of Dairy Products (2) except that incubation was for three days at 25° C. Unless otherwise stated, the skim milk was reconstituted from Bacto skim milk (dehydrated). Eastman tributyrin was used throughout.

Experimental

Acid Recovery

All acids soluble in ether, if present, contribute to the acidity of the ether extract. Since skim milk is the basic medium, lactic and acetic acids assume particular importance.

Solutions of four ether-soluble acids were standardized as follows: acetic acid, 0.0192 *N*; propionic acid, 0.0192 *N*; butyric acid, 0.0197 *N*; and lactic acid, 0.0152 *N*.

Two milliliters of the acid solution to be tested was added to 7 ml. of buffered fresh skim milk. Acidification was with 3.5 ml. of 1.5 *M* phosphoric acid after the addition of tributyrin. In addition a 2.17 ml. mixture of the four acids in buffered fresh skim milk was assayed. The assay data are shown in Table I.

TABLE I

RECOVERY OF ADDED ACIDS

Acid added	Titer	Blank-corrected titer	% recovery
None	0.16	0.00	—
Acetic	0.39	0.23	23
Propionic	0.73	0.57	57
Butyric	1.06	0.90	90
Lactic	0.25	0.09	12
Mixture	0.62	0.46	46

Butyric acid was the most completely recovered, 90%, and lactic acid the least, 12%. The 46% recovery of the mixture of acids was in close agreement with the theoretical 47% calculated from the percentage recovery of the individual acids. Duplicates checked within 2%. This is confirmatory of the results reported in the first paper of this series, in which 90% recovery of butyric acid was obtained.

Nonfat Sources of Acid

Bacterial cultures may contain ether-soluble acids originating from such sources as protein or carbohydrate. It has been shown above that such acids as acetic, propionic, and lactic are recovered in significant proportions during

ether extraction. This is borne out by the data in Table II, which show that for these species the titers before and after the reaction period are high although there is no lipolysis. It is interesting to note that the titer for *S. lactis* is almost identical with the theoretical, 0.50, if the assumptions are made that only lactic acid was produced in the skim milk by this organism, that the titratable acidity was 0.8% expressed as lactic acid, and that 12% of the lactic acid was recovered in the ether layer.

TABLE II

LIPOLYSIS BY TWO ACID-PRODUCING BACTERIA

Organism	Titer		Lipolysis
	Before	After	
S. lactis	0.48	0.51	0.03
E. coli	0.75	0.77	0.02

These data also show that the ether-soluble acids present in the culture before the reaction period do not interfere with the measurement of the acids produced during that period. They are measured in the blank and are eliminated from consideration by subtraction of the titer of the blank from the sample titer.

This leaves the possibility that protein or lactose is a source of acid produced during the reaction period. Therefore, attempts were made to follow protein and lactose breakdown during the reaction period as set forth in Table III.

TABLE III

PROTEIN AND LACTOSE BREAKDOWN DURING THE REACTION PERIOD
(*P. fluorescens*)

	Titer			Change in	
	Before	After	Lipolysis	Amino N	Lactose
Lipolytic activity	0.25	0.65	0.40	—	—
Amino N (mgm./2 ml. culture)	0.060	0.063	—	+0.003	—
Lactose (gm./2 ml. culture)	0.081	0.080	—	—	−0.001

In this experiment there was a 0.003 mgm. increase of amino nitrogen, as determined by the copper method of Albanese and Irby (1), and this value was very closely duplicated in each of a number of trials. This increase is the equivalent of 0.01 ml. of 0.02 *N* sodium hydroxide, which is considered to be within the experimental error of the method. It is concluded that ether-soluble acids are not produced from proteins by *P. fluorescens* during the reaction period.

Determinations by the gravimetric method (3) showed the disappearance of 0.001 gm. of lactose during the reaction period. Assuming complete con-

version of this amount of sugar into lactic acid and 12% extraction of the lactic acid by ether, this amount represents only 0.03 ml. of 0.02 *N* sodium hydroxide as used in this test. Although the gravimetric method of measuring small amounts of lactose is of questionable accuracy because it measures not the actual lactose but the reducing capacity of the solution, dissimilation of lactose by this organism is not to be expected. In the light of further evidence it seems probable that lactose decomposition during the reaction period was of no significance whatsoever.

The strain of *P. fluorescens* used as test organism grew so slowly at 37° C. that significant growth was not to be expected during the reaction period at this temperature. This expectation was justified by plate counts, which did not increase during the 30 min. reaction period. However, lactases might well be active at this temperature. Nevertheless, that lactose was not the source of ether-soluble acid produced during the reaction period is shown in Table IV from which it is seen that neither *P. fluorescens* nor *E. coli*, a lactose

TABLE IV

ACID FORMATION DURING THE REACTION PERIOD

Sample	Tributyrin added	Titer		Lipolysis
		Before	After	
Uninoculated skim milk	—	0.17	0.17	0.00
	+	0.20	0.20	0.00
Inoculated with *P. fluorescens*	−	0.17	0.17	0.00
	+	0.20	0.82	0.62
Inoculated with *E. coli*	−	0.69	0.69	0.00
	+	0.71	0.73	0.02

fermenting bacterium, gave evidence of any lipolysis in the absence of tributyrin. It is concluded, therefore, that the possibility of the production of ether-soluble acid from lactose during the reaction period may be ignored.

TABLE V

VARIATIONS IN REPLICATE CULTURES

Culture number	Titer		Lipolysis
	Before	After	
1	0.16	0.83	0.67
2	0.18	0.93	0.75
3	0.18	0.85	0.67
4	0.19	1.03	0.84
5	0.18	0.93	0.75
6	0.18	0.91	0.73
7	0.18	0.90	0.72
8	0.18	0.94	0.76
9	0.18	0.86	0.68
10	0.19	1.08	0.89
Control	0.18	0.19	0.01

Substrate Concentration

The effect of varying the concentration of tributyrin was studied with a 30 hr. and a 48 hr. culture of *P. fluorescens*. Fig. 1 shows that when lipolysis is very strong it is desirable to use a concentration of the substrate greater than 0.2 ml. Nevertheless, it is thought that 0.2 ml. of tributyrin will prove generally satisfactory.

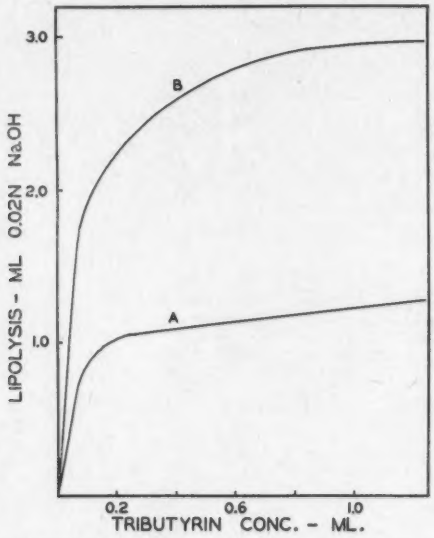


FIG. 1. *Substrate concentration and lipolysis. A = 30 hr. culture. B = 48 hr. culture.*

Variations in Replicate Cultures

Replicate tubes, each containing 10 ml. of sterile skim milk, were inoculated by needle from a 24 hr. slant culture of the test organism. These were incubated at 21° C. for 36 hr. and the lipase activity of each was determined. The lipolytic values of 10 such replicates are shown in Table V. The maximum deviation from the mean lipolysis of 0.75 was $\pm 18.6\%$. This deviation could be expected from probable growth differences in the various replicate cultures.

Amount of Inoculum

The amount of inoculum added to the sterile skim milk before incubation was varied in different ways, using the loop method and pipetting from a water suspension of the test organism. Some variations in lipolysis were noted but these could not be related to the method of inoculation or the amount of inoculum, at least until the latter was large. It is concluded that the loop method is sufficiently accurate for this type of analysis.

pH Range

This method was originally designed to measure milk lipase, which has an optimum activity at approximately pH 8.5. For measuring bacterial lipase

it appeared desirable to know that the method is applicable over a wide pH range.

When the pH during the reaction period was varied, lipolysis by *P. fluorescens* was 2.28 at pH 8.5, 1.37 at pH 7.0, and 1.01 at pH 5.5.

Although it was evident that the method was adaptable over a fairly wide pH range, nevertheless, it seemed desirable to demonstrate its use, not only over a wider pH range, but also with a lipase having an activity optimum on the acid side of neutrality. Therefore, the defatted meat from the dehulled and macerated castor bean (castor bean lipase has an approximate activity optimum at pH 5) was dried, powdered, and suspended in water. Of this suspension 2 ml. was pipetted into 5 ml. of a borate buffer adjusted to the desired pH with normal sodium hydroxide or molar phosphoric acid. A series with varying pH values was so prepared and the pH values determined electrometrically at the start of the reaction period, which in this case was of 60 min. duration. The control titer in each case was determined on a duplicate sample that had been autoclaved for 10 min. at 121° C. before the addition of the buffer. Difficulty was experienced with gel formation in the control samples on shaking with ether. These gels were, however, broken by centrifugalization and offered no further problem. The data from this experiment are presented in Table VI.

TABLE VI

LIPOLYSIS BY CASTOR BEAN LIPASE

pH	Titer		Lipolysis
	Before	After	
2.86	0.24	0.35	0.11
3.10	0.22	0.36	0.14
3.50	0.22	0.73	0.51
3.90	0.22	1.82	1.60
5.95	0.24	1.83	1.59
7.59	0.25	0.32	0.07
8.50	0.24	0.28	0.04
8.72	0.26	0.23	0.00

It is seen that this method measures lipase activity over a sufficiently wide pH range to make it generally applicable in bacteriology.

Discussion

Although extreme accuracy is not claimed for this method, it is believed to offer greater precision than any other method currently available.

It is recognized that, if tributyrin is the only substrate used, tributyrinase may be the only enzyme measured. However, the studies of Collins and Hammer (6) do not point to such narrow specificity of bacterial lipases. In any case the method is adaptable but limited to the study of any substrate that is liquid at room temperature.

The short period during which the enzyme is in contact with the substrate is both an advantage and a disadvantage. It is an advantage in that bacterial growth is eliminated, or nearly so, during this period and extraneous and interfering reactions are held to a minimum. On the other hand, a disadvantage is introduced in that a very slow hydrolysis is not continued long enough to liberate measurable amounts of fatty acid.

References

1. ALBANESE, A. A. and IRBY, VIRGINIA. J. Biol. Chem. 153 : 583-588. 1944.
2. AMERICAN PUBLIC HEALTH ASSOCIATION. Standard methods for the examination of dairy products. 8th ed. Am. Pub. Health Assoc. New York. 1941.
3. ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. Methods of analysis. A.O.A.C. 6th ed. Washington, D.C. 1945.
4. CASTELL, C. H. and BRYANT, L. R. Iowa State Coll. J. Sci. 13 : 313-328. 1939.
5. CASTELL, C. H. and GARRARD, E. H. Can. J. Research, C, 19 : 106-110. 1941.
6. COLLINS, M. A. and HAMMER, B. W. J. Bact. 27 : 473-485. 1934.
7. PETERS, I. I. and NELSON, F. E. J. Bact. 55 : 581-591. 1948.
8. PETERSON, M. H., JOHNSON, M. J., and PRICE, M. V. J. Dairy Sci. 26 : 233-240. 1943.
9. SMITH, L. M., LUBERT, D. J., and THORNTON, H. R. Can. J. Research, F, 27 : 483-490. 1949.
10. TRUSSELL, R. E. and WEED, L. A. J. Bact. 33 : 381-388. 1937.

ESTIMATION OF LIPASE IN DAIRY PRODUCTS

III. LIPASE ACTIVITY IN CULTURES OF MICRO-ORGANISMS AND IN CHEESE[1]

By D. J. Lubert,[2] L. M. Smith,[3] and H. R. Thornton[4]

Abstract

The lipolytic activity in skim milk cultures of micro-organisms representing a number of species and genera were studied by the extraction–titration method described in the preceding paper. No evidence was found of a bacterial lipase having an activity optimum on the acid side of neutrality. No lipase active at approximately pH 5.0 was demonstrated in 20 samples of commercial cheddar cheese of varying age or one sample of blue veined cheese on measurement by the extraction–titration method or by the Peterson *et al.* method. Weak lipolytic activity was found in one sample of blue veined cheese by the extraction–titration method. No lipolytic activity at pH 8.5 was demonstrated by the extraction–titration method in the one sample of cheese tested at this pH.

Introduction

The extraction–titration method of measuring bacterial lipase described in the preceding paper of this series (2) was used to compare the lipolytic activity of 10 species of seven bacterial genera, and some unidentified cultures of bacteria, yeasts, and molds. The study also included 22 samples of cheese.

Methods

Incubation of all cultures and plates was for three days at either 25° C. or 37° C. Lipolysis is expressed as in the preceding paper. Observations were made of the action of giant colonies on Nile blue sulphate agar plates containing 0.5% tributyrin (1).

Titration differences of less than 0.05 are arbitrarily interpreted as representing no lipolysis, 0.05 to 0.1 as questionable lipolysis, 0.1 to 1.0 as weak lipolysis, and over 1.0 as strong lipolysis.

The unidentified "apple" bacterium was a chance isolation that produced an apple odor when grown in pure culture in skim milk. The *A* and *B* cultures were pseudomonads producing a peculiar bitter taste in milk or cream held at low temperatures for long periods but growing well at 27° C.

Aqueous extracts for lipase determinations were prepared by either of two methods from commercial cheese selected at random from various sources.

[1] *Manuscript received July 26, 1949.*
Contribution from the Department of Dairying, University of Alberta, Edmonton, Alberta.
The data contained herein were taken from theses submitted to the University of Alberta by the two senior authors in partial fulfillment of the requirements for the degree of Master of Science. The study was supported by a grant from the Committee on Agricultural Research Grants of the University of Alberta.

[2] *Sessional Instructor in Dairying.*
[3] *Lecturer in Dairying.*
[4] *Professor of Dairying.*

Some of the extracts were prepared by the method of Peterson *et al.* (3) and assayed by the extraction–titration method. The following procedure was used for obtaining the remaining extracts.

Twenty-five grams of the cheese sample was ground in a mortar with sand, and transferred to a 125 ml. Erlenmeyer. This was shaken successively with 50 ml., 25 ml., and 25 ml. of water for 10, 5, and 5 min. respectively. The washings with the suspended material were combined in a 250 ml. centrifuge bottle and centrifuged for 10 min. at 2000 r.p.m. Two-milliliter aliquots were buffered at approximately pH 5.0 and used for the extraction–titration assay. A 10 ml. aliquot of the cheese extract was removed and diluted with water to 25 ml., and 5 ml. of this diluted aliquot was used for lipase determination by the Peterson *et al.* method.

The reaction period in the cheese lipase determinations was of 30 to 60 min. duration while the buffer used was a mixture of phosphoric acid, disodium phosphate, and sodium hydroxide in the proportion necessary to buffer the extract at approximately pH 5.0.

Experimental

Lipolysis by Various Micro-organisms

Scrutiny of Table I reveals that by the criterion of this method nonlipolytic bacteria include *Streptococcus lactis*, *Aerobacter aerogenes*, *Escherichia coli*,

TABLE I

LIPOLYTIC ACTIVITY OF VARIOUS MICRO-ORGANISMS

Arbitrary classification	Organism	Titer		Lipolysis	N.b.s. agar
		Before	After		
No lipolysis	Control	0.18	0.17	0.00	
	S. lactis	0.48	0.51	0.03	−
	A. aerogenes	0.31	0.32	0.01	−
	E. coli	0.75	0.77	0.02	−
	B. subtilis	0.25	0.22	0.00	−
	P. putrefaciens	0.25	0.23	0.00	+
	S. albus	0.24	0.28	0.04	+
	"Apple" bacterium	0.15	0.16	0.01	+
	Yeast	0.19	0.20	0.01	
Questionable lipolysis	*A. faecalis*	0.17	0.23	0.06	+++
	S. aureus	0.23	0.30	0.07	+++
	Mold	0.17	0.23	0.06	
	B 1	0.17	0.23	0.06	
	B 2	0.15	0.22	0.07	
Weak lipolysis	*M. smegmatis*	0.15	0.27	0.12	+++
	A 1	0.16	0.28	0.12	
	A 2	0.17	0.27	0.10	
	A 3	0.16	0.33	0.17	
	B 3	0.16	0.54	0.38	
Strong lipolysis	*P. fluorescens*	0.26	3.90	3.64	++++

Bacillus subtilis, *Pseudomonas putrefaciens*, *Staphylococcus albus*, the "apple odor" and yeast cultures. *Alcaligenes faecalis*, *Staphylococcus aureus*, two of the *B* cultures, and the mold were questionably lipolytic. *Mycobacterium smegmatis* and the *A* and B_3 cultures were weakly lipolytic, while *Pseudomonas fluorescens* exhibited strong lipolysis.

This method and the Nile blue sulphate (N.b.s.) agar method were in general but not complete agreement. Moreover, this method did not demonstrate lipolysis by some organisms well known to be lipolytic, such as *Staphylococcus* (5). The probable explanation is to be found in the short time of contact (30 min.) between the enzyme and the substrate. It should also be noted that in this method lipolysis is effected at pH 8.5, while the Nile blue sulphate agar is at approximately pH 7.0. Enzyme activity may vary markedly within this pH range.

It is evident that this method is not adequate for measuring small differences arising from slow enzyme action or bacterial elaboration of small amounts of enzyme. On the other hand, long reaction periods may, and probably frequently do, lead to interfering extraneous reactions such as ether-soluble acid production.

It is interesting to note that the blank titers of the lactose fermenters, *S. lactis*, *A. aerogenes*, and *E. coli* were high. The probable explanation is that ether-soluble acids, mainly lactic and acetic, were carried over from the skim milk cultures. They are, however, eliminated from the lipolysis figures, which are blank-corrected values. It is to be noted that the blank titer of *A. aerogenes* is lower than that of *E. coli* but no explanation is attempted nor was the relation between the bitter flavor and lipolysis by the *A* and *B* organisms studied.

Relation to pH

In estimating the lipolytic activity of micro-organisms recognition must be given to the possibility that different lipases have differing pH optima. The data in Table II do not indicate such a probability, at least for the six organisms tested. *P. fluorescens* exhibited decreasing lipolysis with decreasing

TABLE II

LIPOLYTIC ACTIVITY OF SOME MICRO-ORGANISMS AT VARIOUS ACIDITIES

Organism	Lipolysis		
	pH 5.5	pH 7.0	pH 8.5
Skim milk	0.00	0.00	0.02
P. fluorescens	1.01	1.37	2.28
S. albus	0.00	0.03	0.05
E. coli	0.00	0.05	0.04
B. subtilis	0.00	0.03	0.07
A. faecalis	0.03	0.06	0.07
Mold	0.00	0.00	0.03

pH, while none of the other species was lipolytic at any pH. It is interesting that *P. fluorescens*, which produces a lipase with optimum activity above pH 8.0, showed considerable lipolytic activity at pH 5.

Cheddar cheese not infrequently becomes rancid and any enzyme causing this defect must be active at the pH of the cheese, which is usually approximately 5.0. In this connection there has not been complete unanimity of opinion as to the role of milk lipase, which is most active on the alkaline side of neutrality (4). Peterson *et al.* (4) regard lipolytic activity in cheese as being bacterially induced and have reviewed the pertinent literature.

Because of the pH of cheddar cheese and because the extraction–titration method is adaptable to measurement of lipase activity at such a low pH, the method was applied to a number of cheese samples. When no lipolysis was demonstrated in eight cheddar and one blue veined cheese, further samples were assayed by the Peterson *et al.* method as well.

It is to be noted (Table III) that, with one exception, there is no suggestion of significant lipase activity by either method of assay. One blue veined cheese exhibited measurable lipolysis as determined by the extraction–titration method.

TABLE III

LIPASE IN CHEESE

Cheese		Reaction time, min.	Extraction–titration method			Peterson et al.
			Titer		Lipolysis	
Sample No.	Age, days		Before	After		Lipase units/ml.
1	15	45	0.21	0.24	0.03	
2	27	45	0.19	0.23	0.04	
3	54	45	0.20	0.24	0.04	
4	91	45	0.25	0.25	0.00	
5	Unknown	30	0.22	0.23	0.01	
6	150	30	0.19	0.19	0.00	
7	150	30	0.20	0.20	0.00	
8	Unknown	60	0.34	0.36	0.02	
9	Unknown	60	0.31	0.32	0.01	
10	Unknown	60	0.41	0.55	0.14	0.01
11	19	60	0.32	0.32	0.00	0.00
12	23	60	0.33	0.33	0.00	0.01
13	27	60	0.33	0.33	0.00	0.00
14	30	60	0.27	0.28	0.01	0.00
15	30	60	0.33	0.33	0.00	0.00
16	35	60	0.27	0.28	0.01	0.00
17	37	60	0.26	0.28	0.02	0.00
18	37	60	0.28	0.27	0.00	0.00
19	40	60	0.29	0.33	0.04	0.00
20	47	60	0.26	0.28	0.02	0.01
21	48	60	0.28	0.28	0.00	0.01
22	54	60	Spoiled	Spoiled	—	0.00

NOTE: *Samples 8 and 10 are blue veined cheese. All others are cheddar cheese.*

There is the possibility of the presence of a lipase active at pH 8.5 but insufficiently active at pH 5 to cause measurable hydrolysis of the tributyrin within 60 min. Cheese number 9, sold as "old Ontario" cheese, was assayed by the extraction–titration method at nine pH values varying from pH 4.67 to pH 9.54. No lipase activity was demonstrated.

Discussion

It would appear that bacterial lipase most vigorously active on the acid side of neutrality is not a common concomitant of bacterial growth. This receives confirmation in the low incidence of rancid cheese.

The theory of a cheese lipase characterized by acid stimulation, whether of bacterial or milk origin, is difficult to reconcile, not only with these studies, but also with common observation of cheese rancidity. The dilatory way in which cheddar cheese acquires this defect lends support to the view that bacterial lipase capable of hydrolyzing the fat in cheese has very weak activity at pH 5. There is no apparent explanation for the variance of these results with those of Peterson *et al.* (3).

References

1. Collins, M. A. and Hammer, B. W. J. Bact. 27 : 473-485. 1934.
2. Lubert, D. J., Smith, L. M., and Thornton, H. R. Can. J. Research, F, 27 : 491-498. 1949.
3. Peterson, M. H., Johnson, M. J., and Price, M. V. J. Dairy Sci. 31 : 31-38. 1948.
4. Peterson, M. H., Johnson, M. J., and Price, M. V. J. Dairy Sci. 31 : 39-46. 1948.
5. Trussell, R. E. and Weed, L. A. J. Bact. 33 : 381-388. 1937.

ESTIMATION OF LIPASE IN DAIRY PRODUCTS

IV. LIPOLYTIC ACTIVITY OF *PSEUDOMONAS FLUORESCENS*[1]

By D. J. Lubert[2], L. M. Smith[3], and H. R. Thornton[4]

Abstract

The lipolytic activity of a strain of *Pseudomonas fluorescens* was investigated. Under the investigational conditions activity was greatest when the reaction medium was at approximately pH 8.9 at the start of the reaction period and when the reaction was carried out at approximately 42° C. The optimum pH for activity by this enzyme was found to be between 8 and 9. This lipase is not specific for tributyrin but hydrolyzes tricaproin and tricaprylin as well, although with decreasing ease. Calcium chloride inhibited rather than enhanced the activity. Lipolytic activity was greater in nutrient broth-base media than in skim milk but the latter was more satisfactory with which to work. Lipolytic activity and fluorescence were not found to be related. Nutrient broth freed of carbohydrate by *Escherichia coli* growth and heat-sterilized stimulated production of fluorescence.

Introduction

Pseudomonas fluorescens had greater lipolytic activity than any of the other bacteria used in these studies. This species is widespread in the waters of the province, occurs almost universally in such products as raw milk and cream, and is frequently concerned with the production of rancidity in these products. A study of the characteristics of the lipase elaborated by this organism was, therefore, considered to be worthwhile.

Methods

Unless otherwise stated, lipolytic activity was measured by the extraction–titration method as described in the second paper of this series (3). The strain of *P. fluorescens* was the same as was used as test organism in the previous studies. It grew well at 21° to 27° C. but very poorly at 37° C. Except as noted, incubation was at 25° C.

Experimental

pH

Lipolysis in a 48 hr. culture (Fig. 1) and a 24 hr. culture (Fig. 2) was determined over a wide range of pH values of the buffered culture at the start of the reaction period. Maximum lipolysis is seen to have been at pH 8.87 in

[1] *Manuscript received July 26, 1949.*
Contribution from the Department of Dairying, University of Alberta, Edmonton, Alberta.
The data contained herein were taken from theses submitted to the University of Alberta by the two senior authors in partial fulfillment of the requirements for the degree of Master of Science. The study was supported by a grant from the Committee on Agricultural Research Grants of the University of Alberta.
[2] *Sessional Instructor in Dairying.*
[3] *Lecturer in Dairying.*
Professor of Dairying.

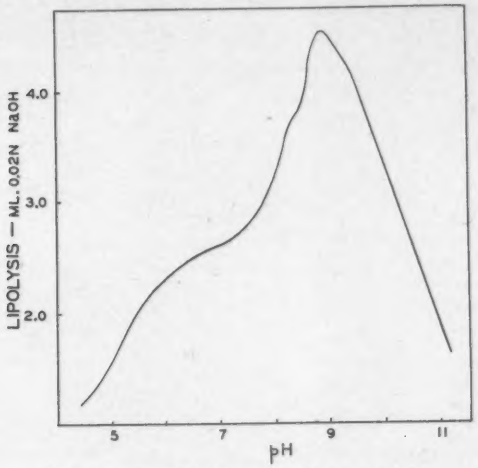


FIG. 1. *pH and lipolysis (48 hr. culture).*

Fig. 1 and 8.92 in Fig. 2. The 24 hr. culture showed very high lipolysis at pH 11 (not shown in Fig. 1). This was believed to be caused by saponification of the tributyrin, which was subsequently found not to be a source of inaccuracy in this method at pH 9 or below. Similar saponification was

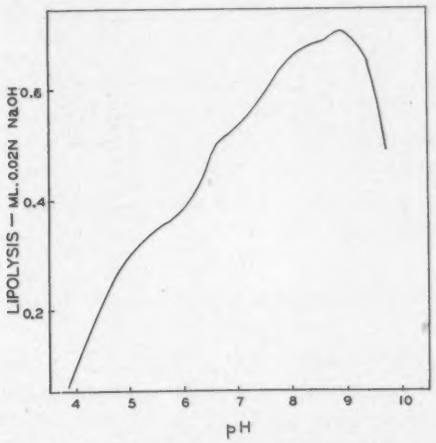


FIG. 2. *pH and lipolysis (24 hr. culture).*

not observed in the 48 hr. culture at pH 11, probably because the pH was rapidly lowered during the reaction period.

It cannot be concluded from these data that pH 8.9 is the optimum for activity by this enzyme, because of possible pH changes in the medium during

the reaction period. This is illustrated in Table I, which gives the pH values of the medium at intervals during the reaction period. Trial 1 represents a culture exhibiting low lipolysis while a higher concentration of the enzyme was present in Trial 2. In each case the pH of the medium decreased as

TABLE I

pH CHANGES DURING THE REACTION PERIOD

Trial	Lipolysis	Reaction time, min.							Total decrease pH units
		0	5	10	15	20	25	30	
1	0.34	8.41	8.37	8.51	8.34	8.36	8.31	8.32	0.09
	0.37	8.54	8.52	8.59	8.49	8.51	8.48	8.48	0.06
	0.35	8.84	8.83	8.83	8.81	8.80	8.78	8.76	0.08
2	2.77	8.42				7.89		7.55	0.87
	2.90	8.68		8.49		8.26		8.00	0.68
	2.82	8.91		8.75		8.55		8.37	0.54

lipolysis progressed. That the changes were related to the liberation of free butyric acid is substantiated by the fact that the addition of 1.2 ml. of 0.92 *N* butyric acid to the usual quantity of buffered medium lowered the pH from 8.81 to 8.3. This concentration of butyric acid is the equivalent of a lipolysis value of 2.9, which is in very close agreement with the results of Trial 2.

The conclusion appears to be justified that this enzyme exhibits maximum activity at pH values between 8 and 9.

Temperature

Lipolysis was determined in the usual manner with an 18 hr. skim milk culture grown at 25° C. The reaction temperature of replicate tubes was varied, using water baths adjusted to 6°, 19°, 26°, 37.5°, 41.5°, 46.5°, and 52° C. The results are graphed in Fig. 3 (Curve A).

This experiment was repeated but a 24 hr. skim milk culture was held at 4.5° C. for seven days before the test. The reaction temperatures were 37°, 42°, 47°, and 51° C. (Fig. 3, Curve B).

It will be observed that the optimum temperature for activity of this lipase is approximately 42° C.

Heat has a double effect on the activity of the enzyme. It stimulates activity by supplying energy and it retards activity by denaturing the enzyme. Below the optimum temperature the first influence dominates and lipolysis increases. Above the optimum temperature the second influence becomes major and lipolysis decreases. The curves suggest that this lipase would be completely inactivated during milk pasteurization (62° C. for 30 min.).

Substrate Specificity

In a study of substrate specificity tricaproin and tricaprylin were substituted for tributyrin. Table II shows that all three triglycerides were attacked

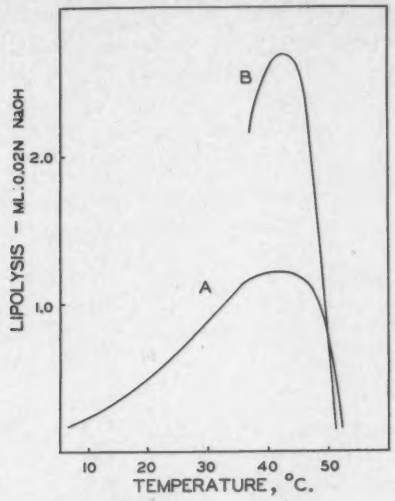


FIG. 3. *Temperature and lipolysis.*

although ease of hydrolysis decreased with increasing molecular weight. This confirms the observations of Collins and Hammer (1) that bacterial lipase is not characterized by narrow substrate specificity.

TABLE II

LIPOLYSIS OF THREE TRIGLYCERIDES

Substrate	Titer		Lipolysis
	Before	After	
Tributyrin	0.23	2.09	1.86
Tricaproin	0.70	1.22	0.52
Tricaprylin	0.34	0.64	0.30

Calcium Chloride

Smith (4) found that 0.25 ml. of 10% calcium chloride per 2 ml. of milk increased the activity of milk lipase. The same concentration of calcium chloride in the reaction mixture tended to retard the activity of *P. fluorescens* (Table III).

TABLE III

INHIBITION BY CALCIUM CHLORIDE

Calcium chloride	Titer		Lipolysis
	Before	After	
Not added	0.32	3.34	3.02
Added	0.32	2.79	2.47

the reaction period. This is illustrated in Table I, which gives the pH values of the medium at intervals during the reaction period. Trial 1 represents a culture exhibiting low lipolysis while a higher concentration of the enzyme was present in Trial 2. In each case the pH of the medium decreased as

TABLE I

PH CHANGES DURING THE REACTION PERIOD

Trial	Lipolysis	Reaction time, min.							Total decrease pH units
		0	5	10	15	20	25	30	
1	0.34	8.41	8.37	8.51	8.34	8.36	8.31	8.32	0.09
	0.37	8.54	8.52	8.59	8.49	8.51	8.48	8.48	0.06
	0.35	8.84	8.83	8.83	8.81	8.80	8.78	8.76	0.08
2	2.77	8.42				7.89		7.55	0.87
	2.90	8.68		8.49		8.26		8.00	0.68
	2.82	8.91		8.75		8.55		8.37	0.54

lipolysis progressed. That the changes were related to the liberation of free butyric acid is substantiated by the fact that the addition of 1.2 ml. of 0.92 *N* butyric acid to the usual quantity of buffered medium lowered the pH from 8.81 to 8.3. This concentration of butyric acid is the equivalent of a lipolysis value of 2.9, which is in very close agreement with the results of Trial 2.

The conclusion appears to be justified that this enzyme exhibits maximum activity at pH values between 8 and 9.

Temperature

Lipolysis was determined in the usual manner with an 18 hr. skim milk culture grown at 25° C. The reaction temperature of replicate tubes was varied, using water baths adjusted to 6°, 19°, 26°, 37.5°, 41.5°, 46.5°, and 52° C. The results are graphed in Fig. 3 (Curve A).

This experiment was repeated but a 24 hr. skim milk culture was held at 4.5° C. for seven days before the test. The reaction temperatures were 37°, 42°, 47°, and 51° C. (Fig. 3, Curve B).

It will be observed that the optimum temperature for activity of this lipase is approximately 42° C.

Heat has a double effect on the activity of the enzyme. It stimulates activity by supplying energy and it retards activity by denaturing the enzyme. Below the optimum temperature the first influence dominates and lipolysis increases. Above the optimum temperature the second influence becomes major and lipolysis decreases. The curves suggest that this lipase would be completely inactivated during milk pasteurization (62° C. for 30 min.).

Substrate Specificity

In a study of substrate specificity tricaproin and tricaprylin were substituted for tributyrin. Table II shows that all three triglycerides were attacked

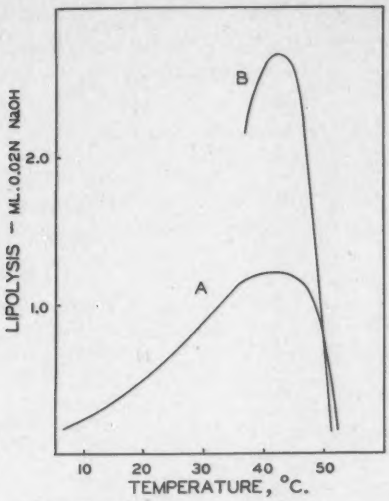


FIG. 3. *Temperature and lipolysis.*

although ease of hydrolysis decreased with increasing molecular weight. This confirms the observations of Collins and Hammer (1) that bacterial lipase is not characterized by narrow substrate specificity.

TABLE II

LIPOLYSIS OF THREE TRIGLYCERIDES

Substrate	Titer		Lipolysis
	Before	After	
Tributyrin	0.23	2.09	1.86
Tricaproin	0.70	1.22	0.52
Tricaprylin	0.34	0.64	0.30

Calcium Chloride

Smith (4) found that 0.25 ml. of 10% calcium chloride per 2 ml. of milk increased the activity of milk lipase. The same concentration of calcium chloride in the reaction mixture tended to retard the activity of *P. fluorescens* (Table III).

TABLE III

INHIBITION BY CALCIUM CHLORIDE

Calcium chloride	Titer		Lipolysis
	Before	After	
Not added	0.32	3.34	3.02
Added	0.32	2.79	2.47

Media

In preliminary experiments 0.7% peptone solution was observed to be unsatisfactory for promoting elaboration of lipase by this organism. Jamieson's (2) modification of Sullivan's "*K*" medium was little better, while skim milk reconstituted from powder Soxhlet-extracted with ether for 24 hr. was the equivalent of the nonextracted skim milk.

The results of a study with modified nutrient broth are given in Table IV. The carbohydrate-free broth was prepared by growing *Escherichia coli* in

TABLE IV

LIPOLYSIS IN VARIOUS MEDIA

Medium	Titer		Lipolysis
	Before	After	
Broth	0.22	0.53	0.31
Broth + 0.05% sodium caseinate	0.17	0.59	0.42
Broth + 0.05% agar	0.17	0.45	0.28
Broth, carbohydrate-free	0.19	0.46	0.27
Broth + 0.15% butter oil	0.17	0.34	0.17
Skim milk	0.20	0.35	0.15
Broth, uninoculated	0.13	0.15	0.02
Skim milk, uninoculated	0.18	0.18	0.00

nutrient broth for 24 hr. at 37° C., after which the culture was filtered through a Berkefeld filter and the filtrate was autoclaved.

Greater lipolysis was obtained with the broth-base media than with the skim milk. This is confirmed by the data in Table V, in which nutrient broth is shown to be superior to skim milk for the encouragement of lipolysis by young cultures but slightly inferior for old cultures. The reason for this is obscure but may be related to growth rates of *P. fluorescens* in different media.

TABLE V

LIPOLYSIS IN THREE MEDIA

Medium	Lipolysis after incubation for		
	24 hr.	48 hr.	96 hr.
Skim milk	0.29	0.86	2.63
Nutrient broth	0.57	1.25	2.13
Carbohydrate-free broth	0.47	1.69	3.10

There is, however, a serious objection to the use of broth-base media in this technique; very considerable difficulty was encountered with gel formation on the addition of ether. Skim milk cultures were found to give more reliable results with more uniform reproducibility. The presence of fat in the medium seems to be unnecessary for lipase elaboration by this organism.

Fluorescence

It was observed that cultures of *P. fluorescens* in the carbohydrate-free broth took on a marked green coloration. The possible relation between lipolysis and fluorescence was, therefore, studied. Unless labeled uninoculated (Table VI), the various media were inoculated with *P. fluorescens*, incubated at 21° C. for three days and given dark-room examination in ultraviolet light.

TABLE VI

FLUORESCENCE PRODUCTION IN VARIOUS MEDIA

Culture medium	Fluorescence	
	Amount	Color
"*K*" medium	++++	Bright green
Nutrient broth	+	Faint green
Nutrient broth inoculated with both *P. fluorescens* and *E. coli*	+	Faint green
Nutrient broth filtrate (Berkefeld filter), autoclaved	+	Faint green
Nutrient broth filtrate (Berkefeld filter), not autoclaved	+	Faint green
Nutrient broth culture of *E. coli*, autoclaved	+	Faint green
Carbohydrate-free broth	+++	Bright green
"*K*" medium, uninoculated	0	None
Nutrient broth, uninoculated	+	Light blue

Since cultures in "*K*" medium are highly fluorescent and poorly lipolytic, broth cultures poorly fluorescent and highly lipolytic, and carbohydrate-free broth cultures highly lipolytic and highly fluorescent, it is concluded that lipolysis and fluorescence are not related.

References

1. COLLINS, M. A. and HAMMER, B. W. J. Bact. 27 : 473-485. 1934.
2. JAMIESON, M. C. Sci. Agr. 22 : 401-409. 1942.
3. LUBERT, D. J., SMITH, L. M., and THORNTON, H. R. Can. J. Research, F, 27 : 491-498. 1949.
4. SMITH, L. M. A method for the estimation of milk lipase. Thesis. Library, University of Alberta. 1949.

SECTION F

INDEX TO VOLUME 27

Authors

SECTION F

INDEX TO VOLUME 27

Subjects

ERRATA

VOLUME 26, 1948

KLEINLOGEL'S RAHMENFORMELN PAPER

Page	Line	In place of	Write
555	Last	$-\frac{h}{6}\cdot 3/2 = -\frac{h}{4}$	$+\frac{h}{6}\cdot 9/4 = +\frac{3}{8}h$
556	2nd	$-\left(\frac{h}{4I_2}+0+\frac{h}{4I_2}\right) = -\frac{h}{2I_2}$	$+\left(\frac{3}{8}\frac{h}{I_1}+0-\frac{h}{8I_1}\right) = +\frac{h}{4I_1}$
	5th	$\frac{7}{8}\frac{h}{I_2}+\frac{1}{12}\frac{I}{I_1}$	$\frac{7}{8}\frac{h}{I_1}+\frac{1}{12}\frac{1}{I_2}$
		$\frac{l}{I_2}$	$\frac{h}{2I_1}+\frac{1}{I_2}$
		$\frac{7}{8}\frac{h}{I_2}+\frac{1}{12}\frac{l}{I_1}$	$\frac{7}{8}\frac{h}{I_1}+\frac{1}{12}\frac{l}{I_2}$
	6th	$-\frac{h}{2I_2}$	$+\frac{h}{4I_1}$
		$-\frac{h}{2I_2}$	$+\frac{h}{4I_1}$
		$-\left(\frac{1}{8}\frac{h}{I_2}+\frac{1}{12}\frac{l}{I_1}\right)$	$-\left(\frac{1}{8}\frac{h}{I_1}+\frac{1}{12}\frac{l}{I_2}\right)$
	9th	$\frac{I_1}{I_2}\cdot\frac{h}{l}$	$\frac{I_2}{I_1}\cdot\frac{h}{l}$
	14th	$-\frac{Ph}{12}\cdot\frac{h}{I_2}-\frac{Ph}{12}\frac{l}{I_1}$	$-\frac{Ph}{12}\frac{h}{I_1}-\frac{Ph}{18}\cdot\frac{l}{I_2}$
	15th	$\frac{Ph}{6}\frac{h}{I_2}$	0
	16th	$\frac{Ph}{12}\frac{h}{I_2}+\frac{Ph}{12}\frac{l}{I_1}$	$+\frac{Ph}{12}\frac{h}{I_1}+\frac{Ph}{18}\frac{l}{I_2}$
	18th	$-\frac{k}{2}$	$+\frac{k}{4}$
		$-\frac{Ph}{12}(k+1)$	$-\frac{Ph}{12}\left(k+\frac{2}{3}\right)$
	19th	$-\frac{k}{2}$	$+\frac{k}{4}$

ERRATA—Continued

KLEINLOGEL'S RAHMENFORMELN PAPER

Page	Line	In place of	Write
556	19th	1	$\frac{1}{2}k + 1$
		$-k/2$	$+k/4$
		$\frac{Ph}{6}k$	0
	20th	$-\frac{k}{2}$	$+\frac{k}{4}$
		$+\frac{Ph}{12}(k+1)$	$+\frac{Ph}{12}(k+2/3)$